NOVEL IMMUNOGENIC LIPOPEPTIDES COMPRISING T-HELPER AND CYTOTOXIC T LYMPHOCYTE (CTL) EPITOPES

Field of the invention

The present invention relates generally to the field of immunology, and more particularly to reagents for generating cellular responses to a peptide immunogen, and methods for using said reagents for enhancing the immune response of a subject, or for the vaccination of a subject. Even more specifically, the present invention relates to novel lipopeptides having enhanced immunogenic activity, specifically an enhanced ability to activate a T cell response to a CD8+ T cell epitope to induce cell mediated immunity against an invading pathogen or tumour cell. The present invention also provides formulations and vaccine compositions comprising said lipopeptides, such as, for example, in combination with a pharmaceutically acceptable carrier or excipient, and methods for making and using the formulations and vaccine compositions of the invention.

Background to the invention

1. General

This specification contains amino acid sequence information prepared using PatentIn Version 3.1, presented herein after the Abstract. Each sequence is identified in the sequence listing by the numeric indicator <210> followed by the sequence identifier (e.g. <210>1, <210>2, etc). The length of each sequence and source organism are indicated by information provided in the numeric indicator fields <211> and <213>, respectively. Sequences referred to in the specification are defined by the term "SEQ ID NO:", followed by the sequence identifier (eg. SEQ ID NO: 1 refers to the sequence designated as <400>1).

As used herein the term "derived from" shall be taken to indicate that a specified integer may be obtained from a particular source albeit not necessarily directly from that source.

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated step or element or integer or group of steps or

elements or integers but not the exclusion of any other step or element or integer or group of elements or integers.

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations or any two or more of said steps or features.

The present invention is not to be limited in scope by the specific examples described herein. Functionally-equivalent products, compositions and methods are clearly within the scope of the invention, as described herein.

All the references cited in this application are specifically incorporated by reference herein.

The present invention is performed without undue experimentation using, unless otherwise indicated, conventional techniques of molecular biology, microbiology, virology, recombinant DNA technology, peptide synthesis in solution, solid phase peptide synthesis, and immunology. Such procedures are described, for example, in the following texts that are incorporated by reference:

1. Sambrook, Fritsch & Maniatis, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratories, New York, Second Edition (1989), whole of Vols I, II, and III;
2. DNA Cloning: A Practical Approach, Vols. I and II (D. N. Glover, ed., 1985), IRL Press, Oxford, whole of text;
3. Oligonucleotide Synthesis: A Practical Approach (M. J. Gait, ed., 1984) IRL Press, Oxford, whole of text, and particularly the papers therein by Gait, pp1-22; Atkinson *et al.,* pp35-81; Sproat *et al.,* pp 83-115; and Wu *et al.*, pp 135-151;
4. Nucleic Acid Hybridization: A Practical Approach (B. D. Hames & S. J. Higgins, eds., 1985) IRL Press, Oxford, whole of text;

5. Animal Cell Culture: Practical Approach, Third Edition (John R.W. Masters, ed., 2000), ISBN 0199637970, whole of text;

6. Immobilized Cells and Enzymes: A Practical Approach (1986) IRL Press, Oxford, whole of text;

7. Perbal, B., A Practical Guide to Molecular Cloning (1984);

8. Methods In Enzymology (S. Colowick and N. Kaplan, eds., Academic Press, Inc.), whole of series;

9. J.F. Ramalho Ortigão, "The Chemistry of Peptide Synthesis" *In:* Knowledge database of Access to Virtual Laboratory website (Interactiva, Germany);

10. Sakakibara, D., Teichman, J., Lien, E. Land Fenichel, R.L. (1976). *Biochem. Biophys. Res. Commun.* **73** 336-342

11. Merrifield, R.B. (1963). *J. Am. Chem. Soc.* 85, 2149-2154.

12. Barany, G. and Merrifield, R.B. (1979) in *The Peptides* (Gross, E. and Meienhofer, J. eds.), vol. 2, pp. 1-284, Academic Press, New York.

13. Wünsch, E., ed. (1974) *Synthese von Peptiden in Houben-Weyls Methoden der Organischen Chemie* (Müler, E., ed.), vol. 15, 4th edn., Parts 1 and 2, Thieme, Stuttgart.

14. Bodanszky, M. (1984) *Principles of Peptide Synthesis*, Springer-Verlag, Heidelberg.

15. Bodanszky, M. & Bodanszky, A. (1984) *The Practice of Peptide Synthesis, Springer-Verlag,* Heidelberg.

16. Bodanszky, M. (1985) *Int. J. Peptide Protein Res.* **25**, 449-474.

17. Handbook of Experimental Immunology, Vols. I-IV (D. M. Weir and C. C. Blackwell, eds., 1986, Blackwell Scientific Publications).

Description of the related art

Immunotherapy or vaccination are attractive for the prophylaxis or therapy of a wide range of disorders, such as, for example, certain infectious diseases, or cancers. However, the application and success of such treatments are limited in part by the poor immunogenicity of the target CTL epitope. Synthetic peptides, representing T cell immunogens elicit only weak immunity when delivered in isolation and as a consequence, are not effective in vaccine compositions. Full-length proteins containing CTL epitopes do not efficiently enter the MHC class I processing pathway. Additionally, CTL epitopes are HLA-restricted and the large

degree of HLA polymorphism in human populations means that CTL-based vaccines may not provide broad coverage to all genotypes within a population.

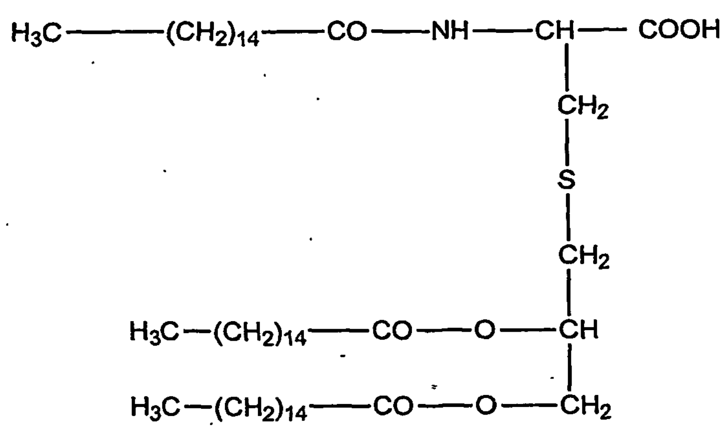
Several techniques are used to enhance the immune response of a subject to a peptide immunogen.

It is known that utilization of an adjuvant formulation that is extrinsic to the peptide immunogen (i.e. it is mixed with the immunogen prior to use), such as, for example, complete Freund's adjuvant (CFA), will enhance the immune response of a subject to a peptide immunogen. However, many of the adjuvants currently available are too toxic for use in humans, or simply ineffective. Moreover, adjuvants of this type require prior formulation with the peptide immunogen immediately before administration, such formulations often having a low solubility or being insoluble.

Lipopeptides, wherein a lipid moiety that is known to act as an adjuvant is covalently coupled to a peptide immunogen, may be capable of enhancing the immunogenicity of an otherwise weakly immunogenic peptide in the absence of an extrinsic adjuvant [Jung *et al., Angew Chem, Int Ed Engl 10*, 872, (1985); Martinon *et al., J Immunol 149,* 3416, (1992); Toyokuni *et al., J Am Chem Soc 116*, 395, (1994); Deprez, *et al., J Med Chem 38,* 459, (1995); and Sauzet *et al., Vaccine 13,* 1339, (1995); BenMohamed *et al., Eur. J. Immunol. 27,* 1242, (1997); Wiesmuller *et al., Vaccine 7*, 29, (1989); Nardin *et al., Vaccine* 16, 590, (1998); Benmohamed, *et al. Vaccine 18*, 2843, (2000); and Obert, *et al., Vaccine 16,* 161, (1998)]. Suitable lipopeptides show none of the harmful side effects associated with adjuvant formulations, and both antibody and cellular responses have been observed against lipopeptides.

Several different fatty acids are known for use in lipid moieties. Exemplary fatty acids include, but are not limited to, palmitoyl, myristoyl, stearoyl and decanoyl groups or, more generally, any C_2 to C_{30} saturated, monounsaturated, or polyunsaturated fatty acyl group is thought to be useful.

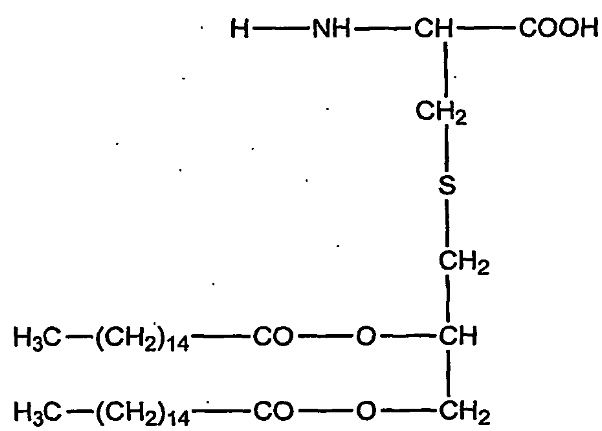
The lipoamino acid N-palmitoyl-S-[2,3-*bis*(palmitoyloxy)propyl]cysteine, also known as Pam_3Cys or Pam_3Cys-OH (Wiesmuller *et al., Z. Physiol.Chem. 364* (1983), p593), is a synthetic version of the N-terminal moiety of Braun's lipoprotein that spans the inner and outer membranes of Gram negative bacteria. Pam_3Cys has the structure of Formula (I):



United States Patent No. 5, 700, 910 to Metzger *et al* (December 23, 1997) describes several N-acyl-S-(2-hydroxyalkyl)cysteines for use as intermediates in the preparation of lipopeptides that are used as synthetic adjuvants, B lymphocyte stimulants, macrophage stimulants, or synthetic vaccines. Metzger *et al.* also teach the use of such compounds as intermediates in the synthesis of Pam_3Cys-OH (Wiesmuller *et al., Z. Physiol. Chem. 364* (1983), p593), and of lipopeptides that comprise this lipoamino acid or an analog thereof at the N-terminus. The lipopeptides are prepared by coupling a lipoamino acid moiety to the peptide moiety during the synthesis process.

Pam_3Cys when coupled to a CTL epitope peptide has been shown to be capable of stimulating virus-specific cytotoxic T lymphocyte (CTL) responses against influenza virus-infected cells (Deres *et al., Nature 342,* 561, 1989) and to elicit protective antibodies against foot-and-mouth disease (Wiesmuller *et al., Vaccine 7*, 29, 1989; United States Patent No. 6,024,964 to Jung *et al.*, February 15, 2000) when coupled to the N-terminus of an appropriate synthetic B cell epitope.

Recently, Pam_2Cys (also known as dipalmitoyl-S-glyceryl-cysteine or S-[2,3-*bis*(palmitoyloxy)propyl]cysteine), an analogue of Pam_3Cys, has been synthesised (Metzger, J. W., A. G. Beck-Sickinger, M. Loleit, M. Eckert, W. G. Bessler, and G. Jung. 1995. *J Pept Sci 1:184.*) and been shown to correspond to the lipid moiety of MALP-2, a macrophage-activating lipopeptide isolated from mycoplasma (Sacht, G., A. Marten, U. Deiters, R. Sussmuth, G. Jung, E. Wingender, and P. F. Muhlradt. 1998. *Eur J Immunol 28:4207*: Muhlradt, P. F., M. Kiess, H. Meyer, R. Sussmuth, and G. Jung. 1998. *Infect Immun 66:4804*: Muhlradt, P. F., M. Kiess, H. Meyer, R. Sussmuth, and G. Jung. 1997. *J Exp Med 185:1951*). Pam_2Cys has the structure of Formula (II):



Pam_2Cys is reported to be a more potent stimulator of splenocytes and macrophages than Pam_3Cys (Metzger *et al., J Pept. Sci 1,* 184, 1995; Muhlradt *et al., J Exp Med 185*, 1951, 1997; and Muhlradt *et al.*, *Infect Immun 66*, 4804, 1998).

Generation of a strong CD8+ T cell response against a given CTL epitope requires the generation of a strong T helper cell response. $CD4^+$ T-helper cells function in cell-mediated immunity (CMI) by secreting sufficient cytokines, such as, for example IL-2, to thereby facilitate the expansion of $CD8^+$ T cells or by interacting with the antigen presenting cell (APC) thereby rendering it more competent to activate $CD8^+$ T cells. Accordingly, it is desirable to administer a CTL epitope in conjunction with at least one T-helper cell epitope (Vitiello *et al., J. Clin. Invest. 95*, 341-349, 1995; Livingston *et al., J. Immunol. 159*, 1383-1392,

1997). These epitopes are recognized by T-helper cells in the context of MHC class II molecules on the surface of the APC.

The CTL epitope or isolated epitope can be administered in conjunction with a large protein having a range of T helper epitopes in order to accommodate the diversity of class II alleles within a population of individuals. Alternatively, promiscuous or permissive T-helper epitope-containing peptides are administered in conjunction with the CTL epitope or epitopes. Promiscuous or permissive T-helper epitope-containing peptides are presented in the context of a vast majority of MHC class II haplotypes, such that they induce strong $CD4^+$ T helper responses in the majority of an outbred human population. Examples of promiscuous or permissive T-helper epitopes are tetanus toxoid peptide, *Plasmodium falciparum* pfg27, lactate dehydrogenase, and HIVgp120 (Contreas *et al., Infect. Immun, 66,* 3579-3590, 1998; Gaudebout *et al., J. A.I.D.S. Human Retrovirol 14*, 91-101, 1997; Kaumaya *et al., J. Mol. Recog. 6,* 81-94, 1993; and Fern and Good *J. Immunol. 148,* 907-913, 1992). Ghosh *et al., Immunol 104*, 58-66, 2001 and International Patent Application No. PCT/AU00/00070 (WO 00/46390) also describe promiscuous T-helper epitopes from the fusion protein of Canine Distemper Virus (CDV-F). Certain promiscuous T-helper epitopes promote strong CTL responses to a given CTL epitope, and can bypass certain haplotype restricted immune responses (Kaumaya *et al., J. Mol. Recog. 6,* 81-94, 1993).

Routinely, a vaccine preparation will comprise a mixture of polypeptides comprising the T-helper cell epitope and CTL epitope, however it is also known to consist of a single polypeptide comprising both the T-helper epitope and the CTL epitope.

Summary of the invention

In work leading up to the present invention, the inventors sought to improve methods for producing highly immunogenic lipopeptides having a lipid moiety and a polypeptide moiety comprising both a T helper epitope and a CTL epitope against which an immune response is desired. The inventors showed that a

highly immunogenic lipopeptide comprising both T-helper and CTL epitopes can be produced by synthesizing a single polypeptide molecule comprising said epitopes with an internal lysine residue or internal lysine analog residue and then coupling the lipid moiety to the side-chain amino group of said internal lysine residue or said internal lysine analog residue, as opposed to the N-terminal attachments described previously. This enables the lipopeptide of the present invention to be synthesized conveniently using a single amino acid chain, thereby requiring no post-synthesis modification to incorporate both epitopes.

By positioning said one or more lysine residue(s) or one or more internal lysine analog residue(s) at predetermined locations within the polypeptide during peptide synthesis, the attachment site of the lipid is readily specified. Positioning of the lipid moiety in the lipopeptide can thus be targeted to enhance the utility of the end-product for vaccine or adjuvant formulations.

The inventors have found that attachment of the lipid moiety via the side-chain epsilon-amino group of an internal lysine residue or the terminal side-chain group of an internal lysine analog residue positioned between the amino acid sequences of the T helper epitope and the CTL epitope, provides an enhancement of dendritic cell maturation when compared to linear structures obtained in which lipid is attached to the N-terminus of the peptide.

One advantage provided by the lipopetides of the present invention is that they are sufficiently immunogenic such that it is generally not necessary to include an extrinsic adjuvant in vaccine formulations comprising these lipopeptides.

The present invention clearly encompasses the attachment of a lipid moiety via the epsilon-amino group of an internal lysine residue or the terminal side-chain group of an internal lysine analog residue present in the amino acid sequence of the T helper epitope or the amino acid sequence of the CTL epitope, the only requirement being that the lipid moiety is not attached to the N-terminus or the C-terminus of the peptide. By "internal" means at a location other than the N-terminus or the C-terminus of a polypeptide comprising a T helper epitope and CTL epitope. As will be known to the skilled person, solubility of a vaccine

containing CTL epitopes is highly desirable for producing vaccine formulations on a commercial basis.

Preferably, the lipid moiety is attached via the epsilon-amino group of a lysine residue or via the terminal side-chain group of a lysine analog residue positioned between the amino acid sequences of the T helper epitope and the CTL epitope.

Optionally, one or more amino acid spacers is added between the T-helper epitope and the CTL epitope, such as, for example, at either side of an internal lysine or lysine analog positioned between said epitopes.

A spacer of any conventional type can also be added between the lipid moiety and the polypeptide moiety. Particularly preferred spacers in this context consist of serine dimers, trimers, teramers, etc. Alternative spacers, such as, for example, arginine dimers, trimers, tetramers, or 6-aminohexanoic acid, are also contemplated for use in this context.

As exemplified herein, the present inventors produced the lipopeptide of the invention by coupling the lipid moiety to an exposed epsilon-amino group of an internal lysine residue in the synthetic peptide moiety. Optionally, a spacer may be added to the exposed epsilon amino group before addition of the lipid moiety.

As will be apparent from the disclosure herein, a lipoamino acid of Formula (III) or (IV) may be added directly to the epsilon amino group of the internal lysine residue or to the terminal side-chain group of an internal lysine analog residue. Lipoamino acids selected from the group consisting of: (i) Pam_2Cys (also known as dipalmitoyl-S-glyceryl-cysteine or S-[2,3-*bis*(palmitoyloxy)propyl]cysteine), (ii) Ste_2Cys (also known as distearoyl-S-glyceryl-cysteine or S-[2,3-*bis*(stearoyloxy)propyl]cysteine), Lau_2Cys (also known as dilauroyl-S-glyceryl-cysteine or S-[2,3-*bis*(lauroyloxy)propyl]cysteine), and Oct_2Cys (also known as dioctanoyl-S-glyceryl-cysteine or S-[2,3-*bis*(octanoyloxy)propyl]cysteine) are also useful.

As exemplified herein, lipopeptides of the invention directed against influenza virus induced a virus-specific CTL response in the absence of external adjuvant, as reflected by their ability to induce potent CTL-mediated virus clearing responses, to induce CD8+ T cell migration to the lungs and to upregulate the surface expression of MHC class II molecules on immature dendritic cells (DC). The enhanced maturation of dendritic cells following administration of the subject lipopeptides is consistent with enhanced T-helper epitope presentation compared to lipopeptides having N-terminally coupled lipid.

As will be clear to those skilled in the art, the nature of the T-helper and CTL epitopes is not critical in the context of the present invention. The novel approach of attaching the lipid moiety to the epsilon amino group of one or more internal lysine residues or lysine analogue residues within the polypeptide portion of the construct has broad application. Accordingly, based on the results presented herein, it will be understood that a wide range of T-helper and CTL epitopes can be used in the lipopeptide constructs.

Brief description of the drawings

Figure 1 is a schematic representation showing the generalized structure of the peptide and lipopeptide constructs used in this study. Peptide structures consisted of a $CD4^{+}$ helper T cell epitope [Th] and a CTL cell epitope [CTL] assembled as tandem linear sequences with a linking internal lysine residue (i.e. [Th]-*Lys*-[CTL]) or without any internal lysine (i.e. [Th]-[CTL]). Lipopeptides were branched structures wherein a lipid moiety was attached through the epsilon-amino group of a lysine residue, *Lys,* situated between the two epitopes at the approximate centre of the molecule (i.e. [Th]-*Lys*(Pam_3Cys-Ser-Ser)-[CTL]; [Th]-*Lys*(Pam_2Cys-Ser-Ser)-[CTL]; or [Th]-*Lys*(Pam_1Cys-Ser-Ser)-[CTL]. In the case of branched constructs, the centrally located lysine residue to which the lipid is attached is denoted in italics, *Lys.* In some cases two serine residues (Ser-Ser) were added between the peptide and lipid moiety. For the lipopeptide Pal_2LysLys[Th]-[CTL], two palmitic acid residues were attached to the alpha and epsilon-amino groups of the N-terminal lysine residue and [Th] was attached to the epsilon-amino group of the penultimate lysine in the amino acid sequence. In

the case of [Th]-*Lys*(Chol$_2$Lys-Ser-Ser)-[CTL], two residues of cholesterol were attached to an N-terminal lysine residue.

Figure 2 is a representation of the primary amino acid sequences of the peptide moieties attached to the lipid moieties for the structures shown in Figure 1. Non-lipidated peptides comprising these amino acid sequences were designated as follows:

(i) [Th] consisting of a CD4$^+$ T-helper epitope from the light chain of influenza virus hemagglutinin as set forth in SEQ ID NO: 1;

(ii) [CTL] consisting of an immunodominant H-2^d-restricted CTL epitope consisting of amino acid residues 147-155 of the nucleoprotein of influenza virus strain A/Puerto Rico/8/34 (PR8;H1N1) as set forth in SEQ ID NO: 2;

(iii) [Th]-[CTL] consisting of a polypeptide having (i) and (ii). The sequence of the assembled peptide is shown in SEQ ID NO: 3;

(iv) [Th]-*Lys*-[CTL] consisting of a polypeptide having (i) and (ii) separated by a lysine residue (bold underlined residue). The sequence of the assembled peptide is shown in SEQ ID NO: 4;

(v) [P25]-*Lys*-[SIINFEKL] consisting of a T-helper epitope from CDV-F protein designated P25 (SEQ ID NO: 20) and a CTL epitope from ovalbumin (SEQ ID NO: 173) separated by a lysine residue (bold underlined residue). The sequence of the assembled peptide is shown in SEQ ID NO: 174;

(vi) [P25]-*Lys*-[LLO91-99] consisting of a T-helper epitope from CDV-F protein designated P25 (SEQ ID NO: 20) and a CTL epitope from *Listeria monocytogenes* (SEQ ID NO: 172) separated by a lysine residue (bold underlined residue). The sequence of the assembled peptide is shown in SEQ ID NO: 175. and

(vii) [P25]-*Lys*-[HCV] consisting of a T-helper epitope from CDV-F protein designated P25 (SEQ ID NO: 20) and a CTL epitope from the core protein of hepatitic C virus (SEQ ID NO: 176) separated by a lysine residue (bold

underlined residue). The sequence of the assembled peptide is shown in SEQ ID NO: 177.

Lipopeptides comprising these amino acid sequences were designated as follows:

(i) [Th]-*Lys*(Pam_3Cys-Ser-Ser)-[CTL] consisting of peptide [Th]-*Lys*-[CTL] (i.e. SEQ ID NO: 4) and a lipid of the Formula (III) conjugated to the epsilon-amino group of the internal lysine (bold underlined residue);

(ii) [Th]-*Lys*(Pam_2Cys-Ser-Ser)-[CTL] consisting of peptide [Th]-*Lys*-[CTL] (i.e. SEQ ID NO: 4) and a lipid of the Formula (IV) conjugated to the epsilon-amino group of the internal lysine (bold underlined residue);

(iii) [P25]-*Lys*(Pam_2Cys-Ser-Ser)-[LLO91-99] consisting of peptide [P25]-*Lys*-[LLO91-99] and a lipid of the Formula (IV) conjugated to the epsilon-amino group of the internal lysine (bold underlined residue) of said peptide;

(iv) [P25]-*Lys*(Pam_2Cys-Ser-Ser)-[SIINFEKL] consisting of peptide [P25]-*Lys*-[SIINFEKL] and a lipid of the Formula (IV) conjugated to the epsilon-amino group of the internal lysine (bold underlined residue) of said peptide;and

(v) [P25]-*Lys*(Pam_2Cys-Ser-Ser)-[HCV] consisting of peptide [P25]-*Lys*-[HCV] and a lipid of the Formula (IV) conjugated to the epsilon-amino group of the internal lysine (bold underlined residue) of said peptide.

Figure 3 is a graphical representation showing the reduced viral load of mice primed with lipopeptides referred to in the legend to Figure 1 and subsequently challenged with influenza virus. Mice were inoculated intranasally with 9 nmol of the lipopeptides [Th]-*Lys*(Pam_3Cys-Ser-Ser)-[CTL] and [Th]-*Lys*(Pam_2Cys-Ser-Ser)-[CTL] in 50 μl PBS (columns 2 and 3, respectively), or for the [Th]-*Lys*-[CTL] peptide in 50 μl PBS (column 1), or with PBS alone (column 4). Peptide and lipopeptide designations are as for the legend to Figure 2. On day 9 post immunization, mice were anesthetized using penthrane and challenged intranasally with 30,000 plaque forming units of influenza virus subtype H3N1 known as A/Memphis/1/71 (Mem 71). Five days later, their lungs were removed

and assayed for the presence of infectious virus by plaque assay on MDCK cells. Each bar represents the geometric mean titre of viral titres from a group of 5 BALB/c mice and error bars represent the standard deviation of the mean. Numbers above the bars represent the percentage reduction in lung viral titre relative to the PBS control.

Figure 4a is a graphical representation showing enhanced lipopeptide-induced viral clearance in immunized mice receiving the lipopeptides referred to in the legend to Figure 2. Mice were inoculated with 9 nmoles of the lipopeptides [Th]-*Lys*(Pam_3Cys-Ser-Ser)-[CTL] and [Th]-*Lys*(Pam_2Cys-Ser-Ser)-[CTL] in 50 µl PBS (columns 2 and 3, respectively), or for the [Th]-*Lys*-[CTL] peptide in 50 µl PBS (column 1), or with PBS alone (column 4). On day 28 post immunization, mice were challenged with 30,000 plaque forming units of Mem 71 virus. Peptide and lipopeptide designations are as for the legend to Figure 2. Data are expressed as the percentage reduction in lung viral titre on day 5 post challenge. Data show enhanced reduction in infectious virus in the lungs of mice immunized with the lipopeptides [Th]-*Lys*(Pam_3Cys-Ser-Ser)-[CTL] (column 2) or [Th]-*Lys*(Pam_2Cys-Ser-Ser)-[CTL] (column 3) compared to peptide alone (column 1) or PBS alone (column 4) at 5 days post-challenge.

Figure 4b is a graphical representation showing enhanced T cell activation in immunized mice receiving the lipopeptides referred to in the legend to Figure 2. Mice were inoculated with 9 nmoles of the lipopeptides [Th]-*Lys*(Pam_3Cys-Ser-Ser)-[CTL] and [Th]-*Lys*(Pam_2Cys-Ser-Ser)-[CTL] in 50 µl PBS (columns 2 and 3, respectively), or for the [Th]-*Lys*-[CTL] peptide in 50 µl PBS (column 1), or with PBS alone (column 4) Immunized mice were killed 9 days post-immunization and a bronchio-alviolar lavage (BAL) performed. Adherent cells were removed by incubation of the BAL sample in a petri dish at 37°C for 1 hour. The non-adherent cells were recovered and stained for CD8 and CD4 expression. The cells were analyzed by flow cytometry. The lymphocyte population was identified based on the forward and side scatter profile and 10,000 lymphocytes were analysed. Data are expressed as the percentage of non-adherent cells in the BAL fluid that are CD8+ lymphocytes. Data show enhanced activation of virus-specific CD8+ T cells in the BAL samples from mice immunized with the lipopeptides [Th]-

Lys(Pam$_3$Cys-Ser-Ser)-[CTL] (column 2) or [Th]-*Lys*(Pam$_2$Cys-Ser-Ser)-[CTL] (column 3) compared to peptide alone (column 1) or PBS alone (column 4) at 5 days post-challenge. Peptide and lipopeptide designations are as for the legend to Figure 2.

Figure 4c is a graphical representation showing enhanced maturation of dendritic cells in response to the lipopeptides referred to in the legend to Figure 2. A line of BALB/c splenic-derived dendritic cells (D1 cells) were incubated overnight with 0.45 nmoles/mL of the peptide [Th]-*Lys*-[CTL] (column 1) or the lipopeptides lipopeptides [Th]-*Lys*(Pam$_3$Cys-Ser-Ser)-[CTL] (column 2) or [Th]-*Lys*(Pam$_2$Cys-Ser-Ser)-[CTL] (column 3) or with medium alone as a negative control (column 4) or lipopolysaccharide as a positive control (LPS; column 5). The percentage of D1 cells expressing high levels of surface MHC class II molecules, and therefore in a mature state, were determined by flow cytometry. Peptide and lipopeptide designations are as for the legend to Figure 2. Data show enhanced expression of MHC class II molecules on the surface of dendritic cells (ie. enhanced dendritic cell maturation) following exposure to the peptides [Th]-*Lys*(Pam$_3$Cys-Ser-Ser)-[CTL] or [Th]-*Lys*(Pam$_2$Cys-Ser-Ser)-[CTL] compared to peptide alone or medium alone.

Figure 5 is a graphical representation showing the induction of pulmonary viral clearing responses in mice inoculated with synthetic immunogens indicated on the x-axis, that each include the $CD4^+$ T-helper epitope set forth in SEQ ID NO: 1 and the *H-2^d*-restricted CTL epitope set forth in SEQ ID NO: 2. Groups of 5 mice were immunised intranasally with 9 nmoles of the specified lipopeptides in PBS. Mice were challenged 28 days after priming with $10^{4.5}$ PFU of Mem71 influenza virus intranasally. Titres of infectious virus in lung homogenates sampled 5 days following challenge were determined by plaque formation on MDCK cell monolayers. Each circle respresents the virus titre of an individual mouse and the line represents the geometric mean titre of the group. The percentage reduction in mean viral titre relative to the PBS control group is shown above each column of data.

Figure 6 is a graphical representation showing accelerated influx of CTL derminant-specific CD8$^+$-T cells into the lungs of lipopeptide-vaccinated mice during virus challenge. Lipopeptides comprised the CD4$^+$ T-helper epitope set forth in SEQ ID NO: 1 and the *H-2^d*-restricted CTL epitope set forth in SEQ ID NO: 2. Groups of three mice were inoculated intranasally with 9 nmole of the indicated lipopeptides intranasally. On day 28 post priming, they were challenged intranasally with $10^{4.5}$ PFU of Mem71 influenza virus intranasally. CTL determinant-specific IFN-gamma-secreting cells were enumerated in the lungs of mice on day 5 post-challenge by an intracellular cytokine production assay. 10,000 CD8+ cells were analysed for each sample. Data represent the mean and standard deviation for each group of mice

Figure 7 is a graphical representation showing show accelerated influx of CTL-determinant-specific CD8 T cells into the lungs in mice inoculated with lipopeptides following viral challenge. Lipopeptides comprised the CD4$^+$ T-helper epitope set forth in SEQ ID NO: 1 and the *H-2^d*-restricted CTL epitope set forth in SEQ ID NO: 2. Mice were inoculated intranasally with 9 nmole of the specified lipopeptides in PBS. Nine days after inoculation mice were challenged intranasally with $10^{4.5}$ PFU of Mem71 influenza virus. On day 5 post infection, CTL-determinant-specific CD8 T cells in the lungs were enumerated by staining the lymphocytes from the lungs with anti-CD8 antibody and with tetrameric MHC class I complexes loaded with the CTL epitope. A total of 30,000 CD8 T cells were analysed.

Figure 8 is a graphical representation showing cytotoxic T cell activity in naïve mice. CTL determinant specific cytotoxicity *in vivo* was measured using syngeneic spleen cells pulsed with the CTL determinant and labelled with high intensity CFSE. Non-pulsed spleen cells labelled with low intensity CFSE were used as a control. A mixture of 15 x 10^6 cells of each target cell population was injected intravenously on day 4 post-infection into naïve mice. The mice were killed 16 hr later and spleens were analysed for the presence of CFSE-high and CFSE-low cell populations by flow cytometry. A total of 1 x 10^6 lymphocytes were analysed for each sample.

Figure 9 is a graphical representation showing cytotoxic T cell activity in lipopeptide-primed mice. A mouse was inoculated intranasally with 9 nmoles [Th]-*Lys*(Pam_2Cys-Ser-Ser)-[CTL] comprising the $CD4^+$ T-helper epitope set forth in SEQ ID NO: 1 and the *H-2^d^*-restricted CTL epitope set forth in SEQ ID NO: 2, in PBS. Mice were challenged with Mem71 on day 28. CTL determinant specific cytotoxicity *in vivo* was measured using syngeneic spleen cells pulsed with the CTL determinant and labelled with high intensity CFSE. Non-pulsed spleen cells labelled with low intensity CFSE were used as a control. A mixture of 15 x 10^6 cells of each target cell population was injected intravenously on day 4 post-infection into the lipopeptide-primed and challenged mice. The mice were killed 16 hr later and spleens were analysed for the presence of CFSE-high and CFSE-low cell populations by flow cytometry. A total of 1 x 10^6 lymphocytes were analysed for each sample.

Figure 10 is a graphical representation showing the ability of various peptide-based immunogens to induce epitope-specific CTL. Lipopeptides comprised the $CD4^+$ T-helper epitope set forth in SEQ ID NO: 1 and the $H\text{-}2^d$-restricted CTL epitope set forth in SEQ ID NO: 2. Groups of three mice were inoculated intranasally with various lipopeptides in PBS and challenged with Mem71 on day 28. In order to analyze CTL determinant specific cytotoxicity *in vivo*, syngeneic spleen cells were pulsed with the CTL determinant and labelled with high intensity CFSE. Antigen-specific lysis was controlled by co-injecting syngeneic spleen cells labelled with low intensity CFSE. A mixture of 15 x 10^6 cells of each target cell population was injected intraveniously on day 4 post-infection. The mice were killed 16 hr later and spleens were analysed for the presence of CFSE-high and CFSE-low cell populations by flow cytometry. A total of 1 x 10^6 lymphocytes were analysed for each sample. Individual mice are represented by the closed squares and the bars represent the geometric mean titre.

Figure 11 is a graphical representation showing induction of interferon-gamma producing cells by lipopeptide. Peptide comprising a T-helper epitope and a CTL epitope of *Listeria monocytogenes* linked via the epsilon amino group of an internal lysine residue positioned between said epitopes to Pam_2Cys (i.e. the peptide [P25]-*Lys*(Pam_2Cys-Ser-Ser)-[LLO91-99] listed in Figure 2 and based

upon SEQ ID NO: 175), or lipopeptide(s) based on this structure in which Pam_2Cys was linked through the epsilon amino group of said lysine, were used to inoculate mice. Five BALB/c mice were inoculated intravenously with bacteria, or subcutaneously with either 9 nmoles of lipidated peptide [P25]-*Lys*(Pam_2Cys-Ser-Ser)-[LLO91-99] or 9 nmoles of non-lipidated peptide [P25]-*Lys*-[LLO91-99] (SEQ ID NO: 175; Figure 2) or phosphate buffered saline (PBS), as indicated on the x-axis. Splenocytes were obtained from the immunized animals and stimulated *in vitro* with either the isolated CTL epitope having the sequence set forth in SEQ ID NO: 172 (open bars) or no antigen (filled bars), and the number of (IFN-gamma) producing cells present was measured 28 days later. The ordinate indicates the number of IFN-gamma producing cells per 1,000,000 splenocytes. Data show enhanced numbers of IFN-gamma producing cells for mice immunized with lipopeptide, indicating an enhanced ability of the lipopeptides to activate T cells relative to non-lipidated peptide.

Figure 12 is a graphical representation showing enhanced protection against *L. monocytogenes* infection for mice immunized with the lipopeptide designated [P25]-*Lys*(Pam_2Cys-Ser-Ser)-[LLO91-99] in Figure 2 (based upon SEQ ID NO: 175). Five 5 BALB/c mice were inoculated intravenously with 1,000 bacteria (column 1), or immunized subcutaneously with PBS (column 2) or 9 nmol [P25]-*Lys*(Pam_2Cys-Ser-Ser)-[LLO91-99] peptide (column 3) or 9 nmol non-lipidated [P25]-*Lys*-[LLO91-99] peptide (SEQ ID NO: 175; column 4), as indicated on the x-axis. Mice were challenged with whole bacteria and the number of colony forming units present in liver was measured 28 days post-challenge (ordinate).

Figure 13 is a graphical representation showing protection against B16 melanoma with lipopeptide vaccination. C57BL/6 mice were vaccinated with 20 nmoles lipidated peptide [P25]-*Lys*(Pam_2Cys-Ser-Ser)-[SIINFEKL] (open circles), non-lipidated peptide [P25]-*Lys*-[SIINFEKL] (open triangles) or with PBS (open squares) subcutaneously in the base of the tail. Mice were then challenged s.c. on the back 14 days later with $2x10^5$ B16-OVA cells (n=6 per group) and tumour growth monitored as described (Anraku, *et al., J Virol. 76*; 3791–3799, 2002).

Figure 14 is a graphical representation showing therapeutic treatment of Lewis Lung tumour with a lipopeptide immunogen, as determinbed by the percentage of animals that remain tumor free following immunization. Mice were injected with $3x10^4$ Lewis Lung tumour cells that had been transfected with ovalbumin and therefore expressed the CTL epitope SIINFEKL [Nelson *et al., J Immunol. 166*: 5557-5566, 2001]. Four days after receiving tumour cells, animals were inoculated with 20 nmoles lipidated peptide [P25]-*Lys*(Pam$_2$Cys-Ser-Ser)-[SIINFEKL] (open circles), non-lipidated peptide [P25]-*Lys*-[SIINFEKL] (open triangles) or with PBS (open squares) subcutaneously in the base of the tail. A second and similar dose of immunogen was administered eleven days after receiving the tumour cells. Animals were monitored for tumour incidence; animals were euthanased when tumour area exceeded 100 mm^2.

Figure 15 is a graphical representation showing therapeutic treatment of Lewis Lung tumour with a lipopeptide immunogen, as determined by measuring survival of animals following immunization. Mice were injected with $3x10^4$ Lewis Lung tumour cells that had been transfected with ovalbumin and therefore expressed the CTL epitope SIINFEKL [Nelson *et al., J Immunol. 166*: 5557-5566, 2001]. Four days after receiving tumour cells, animals were inoculated with 20nmoles lipidated peptide [P25]-*Lys*(Pam$_2$Cys-Ser-Ser)-[SIINFEKL] (open circles), non-lipidated peptide [P25]-*Lys*-[SIINFEKL] (open triangles) or with PBS (open squares) subcutaneously in the base of the tail. A second and similar dose of immunogen was administered eleven days after receiving the tumour cells. Animals were monitored for survival; animals were euthanased when tumour area exceeded 100 mm^2.

Figure 16 is a graphical representation showing the ability of peptide and lipopeptide-based immunogens to up-regulate the expression of MHC class II, CD83 and CD86 on human dendritic cells. Human monocyte-derived dendritic cells were incubated with media alone, LPS (5µg/mL), non-lipidated peptide [P25]-*Lys*-[HCV] (5µg/mL) or lipopeptide [P25]-*Lys*(Pam$_2$Cys-Ser-Ser)-[HCV] (5µg/mL) for 48 hours before staining with FITC-conjugated antibodies for HLA-DR, CD83 and CD86 before analysis by flow cytometry. Histograms are representative of live large granular cells gated on the forward and side scatter

dot plot. Regions of histograms shaded in grey and the given values correspond to the percentage of cells that express high levels of antigen within the analysed populations. The T helper cell epitope was identified from Mobillivirus and has the amino acid sequence KLIPNASLIENCTKAEL (SEQ ID NO: 20); the CTL epitope with the amino acid sequence DLMGYIPLV (SEQ ID NO: 176) is an HLA A2-restricted CTL epitope from the core protein of hepatitis C virus.

Detailed description of the preferred embodiments

Lipopeptides

One aspect of the invention provides an isolated lipopeptide comprising a polypeptide conjugated to one or more lipid moieties wherein:

(i) said polypeptide comprises an amino acid sequence that comprises:
    (a) the amino acid sequence of a T helper cell (Th) epitope and the amino acid sequence of a CTL epitope, wherein said amino acid sequences are different; and
    (b) one or more internal lysine residues or internal lysine analog residues for covalent attachment of each of said lipid moieties via the epsilon-amino group or terminal side-chain group of said lysine or lysine analog; and

(ii) each of said one or more lipid moieties is covalently attached directly or indirectly to an epsilon-amino group of said one or more internal lysine residues or to a terminal side-chain group of said internal lysine analog residues.

As used herein, the term "lipopeptide" means any non-naturally occurring composition of matter comprising one or more lipid moieties and one or more amino acid sequences that are directly or indirectly conjugated, said composition of matter being substantially free of conspecific non-conjugated lipid or protein.

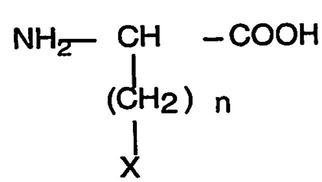
By "directly" means that a lipid moiety and an amino acid sequence are juxtaposed in said lipopeptide (i.e. they are not separated by a spacer molecule).

By "indirectly" means that a lipid moiety and an amino acid sequence are separated by a spacer comprising one or more carbon-containing molecules, such as, for example, one or more amino acid residues. The amino acid sequence may be of any length, constrained by the requirement for functionality of both the T-helper epitope and the CTL epitope.

As used herein, the term "internal lysine residue" means a lysine residue in the polypeptide comprising both the T-helper epitope and the CTL epitope, wherein said lysine is not the N-terminal amino acid residue or the C-terminal residue of said polypeptide. Accordingly, the internal lysine residue may be a C-terminal or N-terminal residue of either the T-helper epitope or the CTL epitope, provided that it is internalized in the polypeptide. This means that the internal lysine residue to which the lipid moiety is attached is a residue that is present in the amino acid sequence of the T helper cell epitope or the amino acid sequence of the CTL epitope. The internal lysine residue may also be distinct from the T-helper epitope or the CTL epitope, in which case it must link these two epitopes of the polypeptide.

Similarly, the term "internal lysine analog residue" means a lysine analog residue in the polypeptide comprising both the T-helper epitope and the CTL epitope, wherein said lysine analog is not the N-terminal amino acid residue or the C-terminal residue of said polypeptide. The crtieria for establishing whether or not a lysine residue is "internal" shall apply *mutatis mutandis* to determing whether or not a lysine analog is internal.

By "lysine analog" is meant a synthetic compound capable of being incorporated into the internal part of a peptide that has a suitable side-group to which the lipid moiety can be coupled, including an amino acid analog or non-naturally occurring amino acid having such an amino side group. Preferred lysine analogs include compounds of the following general Formula (V):



wherein n is an integer from 0 to 3 and wherein X is a terminal side-chain group of said internal lysine analog residue selected from the group consisting of NH, O and S. More preferably, n is an integer having a value from 1 to 3. More preferably, X is an amino group. In a particularly preferred embodiment, the lysine analog is selected from the group consisting of 2,3 diaminopropionic acid (Dpr), 2,4-diaminobutyric acid (Dab) and 2,5-diaminovaleric acid [i.e. ornithine (Orn)].

Those skilled in the art will know the meaning of the term "epsilon-amino group". The term "terminal side-chain group" means a substituent on the side chain of a lysine analog the is distal to the alpha-carbon of said analog, such as, for example, a beta-amino of Dpr, gamma-amino of Dab, or delta-amino of Orn.

Preferably, the lipid moiety is attached via the epsilon amino group of a lysine residue or to a terminal side-chain group of said internal lysine analog residue that is positioned between the amino acid sequences of the T helper epitope and the CTL epitope.

The enhanced ability of the lipopeptides of the invention to elicit a T cell response is reflected by their ability to upregulate the surface expression of MHC class II molecules on immature dendritic cells (DC), particularly D1 cells, and by the enhanced number of $CD8^+$ T cells in tissue samples of immunized animals. In the case of animals immunized using CTLs of a viral pathogen, the enhanced ability of the lipopeptides of the invention to elicit a T cell response is also indicated by the enhanced viral clearance following immunization of animals.

Preferably, the lipopeptides are soluble, more preferably highly soluble.

As will be known to those skilled in the art, the epsilon amino group of lysine is the terminal amino group of the side chain of this amino acid. Use of the terminal side-chain group of the internal lysine or internal lysine analog for cross-linkage to the lipid moiety facilitates the synthesis of the polypeptide moiety as a co-linear amino acid sequence incorporating both the T-helper epitope and the CTL

epitope. There is a clear structural distinction between a lipopeptide having lipid attached via the epsilon amino group of a lysine residue or the terminal side-chain group of a lysine analog, and a lipopeptide having the lipid attached via an alpha amino group of a lysine in the peptide.

Accordingly, it is particularly preferred for at least one internal lysine residue or internal lysine analog to which the lipid moiety is attached to be positioned within the polypeptide moiety so as to separate the immunologically-functional epitopes. For example, the internal lysine residue or internal lysine analog may act as a spacer and/or linking residue between the epitopes. Naturally, wherein the internal lysine or internal lysine analog is positioned between the T-helper epitope and the CTL epitope, the lipid moiety will be attached at a position that is also between these epitopes, albeit forming a branch from the amino acid sequence of the polypeptide. As exemplified herein, a single internal lysine residue is used to separate CTL and T-helper epitopes (eg. SEQ ID No: 4).

The present invention clearly contemplates the nesting of the internal lysine residue or internal lysine analog residue within a third amino acid sequence that does not function as a CTL epitope or T-helper epitope. For example, the internal lysine or internal lysine analog may be conjugated to one or more different amino acid residues.

The epsilon amino group of the internal lysine or terminal side-chain group of an internal lysine analog can be protected by chemical groups which are orthogonal to those used to protect the alpha-amino and side-chain functional groups of other amino acids. In this way, the epsilon amino group or other side-chain group of an internal lysine or lysine analog can be selectively exposed to allow attachment of chemical groups, such as lipid-containing moieties, specifically to the epsilon amino group or side-chain amino group, as appropriate.

For peptide syntheses using using Fmoc chemistry, a suitable orthogonally protected epsilon group of lysine is provided by the modified amino acid residue

Fmoc-*Lys*(Mtt)-OH (*Nα-Fmoc-Nε-4-methyltrityl-L-lysine)*. Similar suitable orthogonally-protected side-chain groups are available for various lysine anlogs contemplated herein, eg. Fmoc-Orn(Mtt)-OH (*Nα-Fmoc-Nδ-4-methyltrityl-L-Ornithine)*, Fmoc-Dab(Mtt)-OH (*Nα-Fmoc-Nγ-4-methyltrityl-L-diaminobutyric acid)* and Fmoc-Dpr(Mtt)-OH (*Nα-Fmoc-Nβ-4-methyltrityl-L-diaminopropionic acid)*. The side-chain protecting group Mtt is stable to conditions under which the Fmoc group present on the alpha amino group of lysine or a lysine analog is removed but can be selectively removed with 1% trifluoroacetic acid in dichloromethane. Fmoc-*Lys*(Dde)-OH (*Nα-Fmoc-Nε-1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl-L-lysine*) or Fmoc-*Lys*(ivDde)-OH (*Nα-Fmoc-Nε-1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)-3-methylbutyl-L-lysine*) can also be used in this context, wherein the Dde side-chain protecting groups is selectively removed during peptide synthesis by treatment with hydrazine.

For peptide syntheses using Boc chemistry, Boc-*Lys*(Fmoc)-OH can be used. The side-chain protecting group Fmoc can be selectively removed by treatment with piperidine or DBU (1,8-Diazabicyclo[5.4.0]undec-7-ene) but remain in place when the Boc group is removed from the alpha terminus using trifluoroacetic acid.

Preferably, the T helper epitope and CTL epitope are separated by at least one or two or three or four or five amino acid residues including a single internal lysine residue or internal lysine analog residue.

The present invention clearly contemplates the addition of multiple lipid moieties to the polypeptide moiety. For example, the polypeptide may include multiple internal lysine residues and/or multiple internal lysine analogs. Steric hindrance may occur in the addition of lipid if multiple internal lysines or multiple lysine analogs are positioned more closely together, thereby producing a mixture of end-products, or a reduced yield.

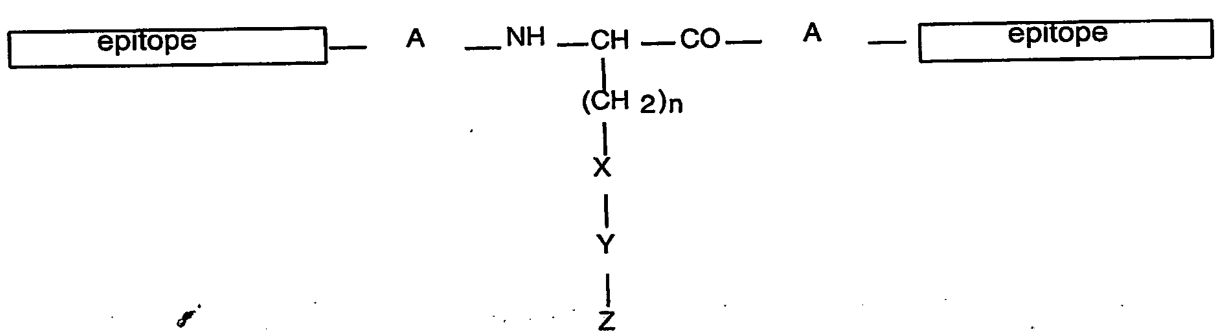
Relevant to this consideration is the fact that it is not necessary for the entire amino acid sequence comprising the T-helper epitope or the entire amino acid sequence comprising the CTL epitope to have an immune function. Accordingly,

the said amino acid sequences, whilst comprising said epitopes may have additional sequence not possessing T-helper cell activity or a CTL epitope. Where such additional sequences include one or more internal lysine or lysine analog residues, the terminal side-chain groups of such residues may serve as attachment sites for the lipid moiety. Naturally, it is essential to retain both T-helper function and CTL epitope function.

The positioning of the internal lysine residue or internal lysine analog for attachment of the lipid moiety should also be selected such that attachment of the lipid moiety does not interfere with the immune function of the T-helper epitope or the CTL epitope in a subject to whom the lipopeptide is administered. For example, depending upon the selection of lipid moiety, the attachment of said lipid within the CTL epitope may sterically hinder CTL epitope presentation.

A generalized preferred form of the lipopeptide of the invention, wherein the internal lysine or internal lysine analog is positioned between the T-helper and CTL epitopes is provided by the general Formula (VI).

Formula (VI):



wherein:

epitope is a T-helper epitope or CTL epitope;

A is either present or absent and consists of an amino acid spacer of about 1 to about 6 amino acids in length;

n is an integer having a value of 1, 2, 3, or 4;

X is a terminal side-chain group selected from the group consisting of NH, O and S and preferably consisting of NH;

Y is either present of absent and consists of an amino acid spacer of about 1 to about 6 amino acids in length, wherein it is preferred for said amino acid to be serine; and

Z is a lipid moiety, preferably Pam_2Cys or Pam_3Cys.

The T-helper epitope is any T-helper epitope known to the skilled artisan for enhancing an immune response in a particular target subject (i.e. a human subject, or a specific non-human animal subject such as, for example, a rat, mouse, guinea pig, dog, horse, pig, or goat). Preferred T-helper epitopes comprise at least about 10-24 amino acids in length, more generally about 15 to about 20 amino acids in length.

Promiscuous or permissive T-helper epitopes are particularly preferred as these are readily synthesized chemically and obviate the need to use longer polypeptides comprising multiple T-helper epitopes.

Examples of promiscuous or permissive T-helper epitopes suitable for use in the lipopeptides of the present invention are selected from the group consisting of:

(i) a rodent or human T-helper epitope of tetanus toxoid peptide (TTP), such as, for example amino acids 830-843 of TTP (Panina-Bordignon *et al., Eur. J. Immun. 19*, 2237-2242, 1989);

(ii) a rodent or human T-helper epitope of *Plasmodium falciparum* pfg27;

(iii) a rodent or human T-helper epitope of lactate dehydrogenase;

(iv) a rodent or human T-helper epitope of the envelope protein of HIV or HIVgp120 (Berzofsky *et al., J. Clin. Invest. 88*, 876-884, 1991);

(v) a synthetic human T-helper epitope (PADRE) predicted from the amino acid sequence of known anchor proteins (Alexander *et al., Immunity 1*, 751-761, 1994);

(vi) a rodent or human T-helper epitope of measles virus fusion protein (MV-F; Muller *et al., Mol. Immunol. 32*, 37-47, 1995; Partidos *et al., J. Gen. Virol., 71*, 2099-2105, 1990);

(vii) a T-helper epitope comprising at least about 10 amino acid residues of canine distemper virus fusion protein (CDV-F) such as, for example, from

amino acid positions 148-283 of CDV-F (Ghosh *et al., Immunol. 104,* 58-66, 2001; International Patent Publication No. WO 00/46390);

(viii) a human T-helper epitope derived from the peptide sequence of extracellular tandem repeat domain of MUC1 mucin (US Patent Application No. 0020018806);

(ix) a rodent or human T-helper epitope of influenza virus hemagglutinin (IV-H) (Jackson *et al. Virol. 198*, 613-623, 1994); and

(x) a bovine or camel T-helper epitope of the VP3 protein of foot and mouth disease virus (FMDV-0_1 Kaufbeuren strain), comprising residues 173 to 176 of VP3 or the corresponding amino acids of another strain of FMDV.

As will be known to those skilled in the art, a T-helper epitope may be recognised by one or more mammals of different species. Accordingly, the designation of any T-helper epitope herein is not to be considered restrictive with respect to the immune system of the species in which the epitope is recognised. For example, a rodent T-helper epitope can be recognised by the immune system of a mouse, rat, rabbit, guinea pig, or other rodent, or a human or dog.

More preferably, the T-helper epitope will comprise an amino acid sequence selected from the group consisting of:

(i) ALNNRFQIKGVELKS from IV-H (SEQ ID NO: 1);
(ii) GALNNRFQIKGVELKS from IV-H (SEQ ID NO: 14);
(iii) LSEIKGVIVHRLEGV from MV-F (SEQ ID NO: 15);
(iv) TAAQITAGIALHQSNLN from CDV-F (SEQ ID NO: 16);
(v) IGTDNVHYKIMTRPSHQ from CDV-F (SEQ ID NO: 17);
(vi) YKIMTRPSHQYLVIKLI from CDV-F (SEQ ID NO: 18);
(vii) SHQYLVIKLIPNASLIE from CDV-F (SEQ ID NO: 19);
(viii) KLIPNASLIENCTKAEL from CDV-F (SEQ ID NO: 20);
(ix) LIENCTKAELGEYEKLL from CDV-F (SEQ ID NO: 21);
(x) AELGEYEKLLNSVLEPI from CDV-F (SEQ ID NO: 22);
(xi) KLLNSVLEPINQALTLM from CDV-F (SEQ ID NO: 23);
(xii) EPINQALTLMTKNVKPL from CDV-F (SEQ ID NO: 24);
(xiii) TLMTKNVKPLQSLGSGR from CDV-F (SEQ ID NO: 25);
(xiv) KPLQSLGSGRRQRRFAG from CDV-F (SEQ ID NO: 26);

(xv) SGRRQRRFAGVVLAGVA from CDV-F (SEQ ID NO: 27);
(xvi) FAGVVLAGVALGVATAA from CDV-F (SEQ ID NO: 28);
(xvii) GVALGVATAAQITAGIA from CDV-F (SEQ ID NO: 29);
(xviii) GIALHQSNLNAQAIQSL from CDV-F (SEQ ID NO: 30);
(xix) NLNAQAIQSLRTSLEQS from CDV-F (SEQ ID NO: 31);
(xx) QSLRTSLEQSNKAIEEI from CDV-F (SEQ ID NO: 32);
(xxi) EQSNKAIEEIREATQET from CDV-F (SEQ ID NO: 33);
(xxii) SSKTQTHTQQDRPPQPS from CDV-F (SEQ ID NO: 34);
(xxiii) QPSTELEETRTSRARHS from CDV-F (SEQ ID NO: 35);
(xxiv) RHSTTSAQRSTHYDPRT from CDV-F (SEQ ID NO: 36);
(xxv) PRTSDRPVSYTMNRTRS from CDV-F (SEQ ID NO: 37);
(xxvi) TRSRKQTSHRLKNIPVH from CDV-F (SEQ ID NO: 38);
(xxvii) TELLSIFGPSLRDPISA from CDV-F (SEQ ID NO: 39);
(xxviii) PRYIATNGYLISNFDES from CDV-F (SEQ ID NO: 40);
(xxix) CIRGDTSSCARTLVSGT from CDV-F (SEQ ID NO: 41);
(xxx) DESSCVFVSESAICSQN from CDV-F (SEQ ID NO: 42);
(xxxi) TSTIINQSPDKLLTFIA from CDV-F (SEQ ID NO: 43);
(xxxii) SPDKLLTFIASDTCPLV from CDV-F (SEQ ID NO: 44);
(xxxiii) STAPPAHGVTSAPDTRAPGSTAPP from MUC-1 (SEQ ID NO: 45);
(xxxiv) GVTSAPDTRPAPGSTASSL from MUC-1 (SEQ ID NO: 46);
(xxxv) GVTSAPDTRPAPGSTASL from MUC-1(SEQ ID NO: 47);
(xxxvi) TAPPAHGVTSAPDTRPAPGSTAPPKKG from MUC-1 (SEQ ID NO: 48);
(xxxvii) STAPPAHGVTSAPDTRPAPGSTAPPK of MUC-1 (SEQ ID NO: 49);
(xxxviii) GVAE from FMDV-VP3 protein (SEQ ID NO: 50);
(xxxix) TASGVAETTN of FMDV-VP3 (residues 170 to 179) (SEQ ID NO: 51); and
(xl) TAKSKKFPSYTATYQF from FMDV (SEQ ID NO: 52).

The T-helper epitopes disclosed herein are included for the purposes of exemplification only. Using standard peptide synthesis techniques known to the skilled artisan, the T-helper epitopes referred to herein are readily substituted for a different T-helper epitope to adapt the lipopeptide of the invention for use in a different species. Accordingly, additional T-helper epitopes known to the skilled person to be useful in eliciting or enhancing an immune response in a target species are not to be excluded.

Additional T-helper epitopes may be identified by a detailed analysis, using *in vitro* T-cell stimulation techniques of component proteins, protein fragments and peptides to identify appropriate sequences (Goodman and Sercarz, *Ann. Rev. Immunol., 1*, 465, (1983); Berzofsky, *In*: "The Year in Immunology, Vol. 2" page 151, Karger, Basel, 1986; and Livingstone and Fathman, *Ann. Rev. Immunol., 5*, 477, (1987)).

The CTL epitope is conveniently derived from the amino acid sequence of an immunogenic protein, lipoprotein, or glycoprotein of a virus, prokaryotic or eukaryotic organism, including but not limited to a CTL epitope derived from a mammalian subject or a bacterium, fungus, protozoan, or parasite that infects said subject. Mimotopes of the CTL epitopes are specifically included within the scope of the invention.

The CTL epitope will be capable of eliciting a T cell response when administered to a mammal, preferably by activating CD8+ T cells specific for the epitope or antigen from which the epitope was derived, and more preferably, by inducing cell mediated immunity against the pathogen or tumour cell from which the epitope is derived.

Shorter CTL epitopes are preferred, to facilitate peptide synthesis. Preferably, the length of the CTL epitope will not exceed about 30 amino acids in length. More preferably, the CTL epitope sequence consists of about 25 amino acid residues or less, and more preferably less than 20 amino acid residues, and even more preferably about 8-12 amino acid residues in length.

Preferred CTL epitopes from parasites are those associated with leishmania, malaria, trypanosomiasis, babesiosis, or schistosomiasis, such as, for example a CTL epitope of an antigen of a parasite selected from the group consisting of: *Plasmodium falciparum; Circumsporozoa; Leishmania donovani; Toxoplasma gondii; Schistosoma mansoni; Schistosoma japonicum; Schistosoma hematobium;* and *Trypanosoma brucei.*

Particularly preferred CTL epitopes of *P. falciparum* are derived from an antigen selected from the group consisting of: circumsporozoite protein (CSP), sporozoite surface protein 2 (PfSSP2), liver stage antigen 1 (LSA1), merozoite surface protein 1 (MSP1), serine repeat antigen (SERA), and AMA-1 antigen (Amante, *et al. J. Immunol. 159*, 5535-5544, 1997; Chaba *et al. Int. J. Immunopharm. 20*, 259-273, 1998; Shi *et al., Proc. Natl Acad. Sci (USA) 96,* 1615-1620, 1999; Wang *et al. Science 282*, 476-479, 1998; and Zevering *et al. Immunol. 94*, 445-454, 1998). Particularly preferred CTL epitopes of *L. donovani* are derived from the Repetitive Peptide (Liew *et al., J. Exp. Med. 172,* 1359 (1990)). Particularly preferred CTL epitopes of *T. gondii* are derived from the P30 surface protein (Darcy *et al., J. Immunol. 149,* 3636 (1992)). Particularly preferred CTL epitopes of *S. mansoni* are derived from the Sm-28GST antigen (Wolowxzuk *et al., J. Immunol 146*:1987 (1991)).

Preferred virus-specific CTL epitopes are derived from Rotaviruses, Herpes viruses, Corona viruses, Picornaviruses (eg. *Apthovirus*), Respiratory Synctial virus, Influenza Virus, Parainfluenza virus, Adenovirus, Pox viruses, Bovine herpes virus Type I, Bovine viral diarrhea virus, Bovine rotaviruses, Canine Distemper Virus (CDV), Foot and Mouth Disease Virus (FMDV), Measles Virus (MV), Human Immunodeficiency Viruses (HIV), Feline Immunodeficiency Viruses (FIV), Epstein-Barr virus (EBV), Human Cytomegalovirus (HCMV), or hepatitis viruses, and the like.

Particularly preferred CTL epitopes of HIV-1 are derived from the *env, gag,* or *pol* proteins. Particularly preferred CTL epitopes of influenza virus are derived from the nucleoprotein (Taylor *et al., Immunogenetics 26*, 267 (1989); Townsend *et al., Nature 348*, 674(1983)), matrix protein (Bednarek *et al., J. Immunol. 147*, 4047 (1991)) or polymerase protein (Jameson *et al., J. Virol. 72*, 8682-8689, 1998; and Gianfrani *et al., Human Immunol. 61*, 438-452, 2000). Particularly preferred CTL epitopes of Lymphocytic choriomeningitis virus (LCMV) are derived from glycoprotein-1 antigen (Zinkernagel *et al. Nature 248,* 701-702, 1974). Particularly preferred CTL epitopes of cytomegalovirus are derived from an antigen selected from the group consisting of: of pp28, pp50, pp65, pp71, pp150, gB, gH, IE-1, IE-2, US2, US3, US6, US11, and UL18 (eg. Diamond, USSN 6,074,645, June 13,

2000; Longmate *et al., Immunogenet. 52*, 165-173, 2000; Wills *et al., J. Virol. 70*, 7569-7579, 1996; Solache *et al., J. Immunol. 163*, 5512-5518, 1999; Diamond *et al., Blood 90*, 1751-1767, 1997; Kern *et al., Nature Med. 4,* 975-978, 1998; Weekes *et al., J. Virol. 73*, 2099-2108, 1999; Retière *et al., J. Virol. 74*, 3948-3952, 2000; and Salquin *et al., Eur. J. Immunol. 30*, 2531-2539, 2000). Particularly preferred CTL epitopes of Measles Virus are derived from the fusion glycoprotein (MV-F) and particularly from residues 438-446 thereof (Herberts *et al. J. Gen Virol. 82*, 2131-2142, 2001). Particularly preferred epitopes from Epstein-Barr virus (EBV) are derived from a latent nuclear antigen (EBNA) or latent membrane protein (LMP) of EBV, such as, for example, EBNA 2A, EBNA 3A, EBNA 4A, or EBNA 14a from EBV type A; EBNA 2B, EBNA 3B, EBNA 4B, or EBNA 14b from EBV type B; LMP1; or LMP2 (International Patent Application No. PCT/AU95/00140 published Sep. 16, 1995; International Patent Application No. PCT/AU97/00328 published Nov. 24, 1997; and International Patent Application No. PCT/AU98/00531 published Jan. 10, 1998).

Preferred bacteria-specific CTL epitopes are derived from *Pasteurella, Actinobacillus, Haemophilus, Listeria monocytogenes, Mycobacterium tuberculosis, Staphylococcus, Neisseria gonorrhoeae, Helicobacter pylori, Streptococcus pneumoniae, Salmonella enterica, E. coli, Shigella,* and the like.

Suitable bacterial CTL epitopes include, for example, those CTL epitopes derived from the *Mycobacterium tuberculosis* 65Kd protein (Lamb *et al., EMBO J., 6,* 1245 (1987)); *M. tuberculosis* ESAT-6 protein (Morten *et al., Infect. Immun. 66,* 717–723, 1998); *Staphylococcus aureus* nuclease protein (Finnegan *et al., J. Exp. Med. 164*, 897 (1986)); *Escherichia coli* heat stable enterotoxin (Cardenas *et al., Infect. Immunity 61,* 4629 (1993)); and *Escherichia coli* heat labile enterotoxin (Clements *et al., Infect. Immunity 53*, 685 (1986)).

Preferred CTL epitopes from mammalian subjects are derived from and/or capable of generating T cell responses against a tumor CTL antigen. Tumor-specific CTL epitopes are usually native or foreign CTL epitopes, the expression of which is correlated with the development, growth, presence or recurrence of a tumor. In as much as such CTL epitopes are useful in differentiating abnormal

from normal tissue, they are useful as a target for therapeutic intervention. Such CTL epitopes are well known in the art. Indeed, several examples are well-characterized and are currently the focus of great interest in the generation of tumor-specific therapies. Non-limiting examples of tumor CTL epitopes are derived from carcinoembryonic antigen (CEA), prostate specific antigen (PSA), melanoma antigen (MAGE, BAGE, GAGE), and mucins, such as MUC-1.

Preferred CTL epitopes for administering to a cancer patient are derived from a protein that induces cancer, such as, for example, an oncoprotein (e.g., p53, ras etc.).

In a particularly preferred embodiment, the CTL epitope will comprise or consist of an amino acid sequence selected from the group consisting of:

(i) TYQRTRALV from the NP of PR8 virus (SEQ ID NO: 2);
(ii) KPKDELDYENDIEKKICKMEKCS of *P. falciparum* CSP (SEQ ID NO: 53);
(iii) DIEKKICKMEKCSSVFNVVNS from *P. falciparum* CSP (SEQ ID NO: 54);
(iv) KPIVQYDNF from *P. falciparum* LSA1 (SEQ ID NO: 55);
(v) GISYYEKVLAKYKDDLE from *P. falciparum* MSP1 (SEQ ID NO: 56);
(vi) EFTYMINFGRGQNYWEHPYQKS of *P. falciparum* AMA-1 (SEQ ID NO: 57);
(vii) DQPKQYEQHLTDYEKIKEG from *P. falciparum* AMA-1 (SEQ ID NO: 58);
(viii) NMWQEVGKAM from HIV-1 *env* protein (SEQ ID NO: 59);
(ix) APTKAKRRVV from HIV-1 *env* protein (SEQ ID NO: 60);
(x) CTRPNNNTRK from HIV-1 *env* protein (SEQ ID NO: 61);
(xi) TVYYGVPVWK from HIV-1 *env* protein (SEQ ID NO: 62);
(xii) RPVVSTQLL from HIV-1 *env* protein (SEQ ID NO: 63);
(xiii) SLYNTVATLY from HIV-1 *gag* protein (SEQ ID NO: 64);
(xiv) ELRSLYNTVA from HIV-1 *gag* protein (SEQ ID NO: 65);
(xv) KIRLRPGGKK from HIV-1 *gag* protein (SEQ ID NO: 66);
(xvi) IRLRPGGKKK from HIV-1 *gag* protein (SEQ ID NO: 67);
(xvii) RLRPGGKKK from HIV-1 *gag* protein (SEQ ID NO: 68);
(xviii) GPGHKARVLA from HIV-1 *gag* protein (SEQ ID NO: 69);
(xix) SPIETVPVKL from HIV-1 *pol* protein (SEQ ID NO: 70);
(xx) ILKEPVHGVY from HIV-1 *pol* protein (SEQ ID NO: 71);
(xxi) AIFQSSMTK from HIV-1 *pol* protein (SEQ ID NO: 72);

(xxii) SPAIFQSSMT from HIV-1 *pol* protein (SEQ ID NO: 73);
(xxiii) QVRDQAEHLK from HIV-1 *pol* protein (SEQ ID NO: 74);
(xxiv) GPKVKQWPLT from HIV-1 *pol* protein (SEQ ID NO: 75);
(xxv) TYQRTRALV from influenza virus nucleoprotein (SEQ ID NO: 76);
(xxvi) TYQRTRALVRTGMDP from influenza nucleoprotein (SEQ ID NO: 77);
(xxvii) IASNENMDAMESSTL from influenza virus nucleoprotein (SEQ ID NO: 78);
(xxviii)KAVYNFATM from LCMV gp1 (SEQ ID NO: 79);
(xxix) QVKWRMTTL from EBV (SEQ ID NO: 80);
(xxx) VFSDGRVAC from EBV (SEQ ID NO: 81);
(xxxi) VPAPAGPIV from EBV (SEQ ID NO: 82);
(xxxii) TYSAGIVQI from EBV (SEQ ID NO: 83);
(xxxiii)LLDFVRFMGV from EBV (SEQ ID NO: 84);
(xxxiv) QNGALAINTF from EBV (SEQ ID NO: 85);
(xxxv) VSSDGRVAC from EBV (SEQ ID NO: 86);
(xxxvi) VSSEGRVAC from EBV (SEQ ID NO: 87);
(xxxvii) VSSDGRVPC from EBV (SEQ ID NO: 88);
(xxxviii) VSSDGLVAC from EBV (SEQ ID NO: 89);
(xxxix) VSSDGQVAC from EBV (SEQ ID NO: 90);
(xl) VSSDGRVVC from EBV (SEQ ID NO: 91);
(xli) VPAPPVGPIV from EBV (SEQ ID NO: 92);
(xlii) VEITPYEPTG from EBV (SEQ ID NO: 93);
(xliii) VEITPYEPTW from EBV (SEQ ID NO: 94);
(xliv) VELTPYKPTW from EBV (SEQ ID NO: 95);
(xlv) RRIYDLIKL from EBV (SEQ ID NO: 96);
(xlvi) RKIYDLIEL from EBV (SEQ ID NO: 97);
(xlvii) PYLFWLAGI. from EBV (SEQ ID NO: 98);
(xlviii) TSLYNLRRGTALA from EBV (SEQ ID NO: 99);
(xlix) DTPLIPLTIF from EBV (SEQ ID NO: 100);
(l) TVFYNIPPMPL from EBV (SEQ ID NO: 101);
(li) VEITPYKPTW from EBV (SEQ ID NO: 102);
(lii) VSFIEFVGW from EBV (SEQ ID NO: 103);
(liii) FRKAQIQGL from EBV (SEQ ID NO: 104);
(liv) FLRGRAYGL from EBV (SEQ ID NO: 105);
(lv) QAKWRLQTL from EBV (SEQ ID NO: 106);

(lvi) SVRDRLARL from EBV (SEQ ID NO: 107);
(lvii) YPLHEQHGM from EBV (SEQ ID NO: 108);
(lviii) HLAAQGMAY from EBV (SEQ ID NO: 109);
(lix) RPPIFIRRL from EBV (SEQ ID NO: 110);
(lx) RLRAEAGVK from EBV (SEQ ID NO: 111);
(lxi) IVTDFSVIK from EBV (SEQ ID NO: 112);
(lxii) AVFDRKSDAK from EBV (SEQ ID NO: 113);
(lxiii) NPTQAPVIQLVHAVY from EBV (SEQ ID NO: 114);
(lxiv) LPGPQVTAVLLHEES from EBV (SEQ ID NO: 115);
(lxv) DEPASTEPVHDQLL from EBV (SEQ ID NO: 116);
(lxvi) RYSIFFDY from EBV (SEQ ID NO: 117);
(lxvii) AVLLHEESM from EBV (SEQ ID NO: 118);
(lxviii) RRARSLSAERY from EBV (SEQ ID NO: 119);
(lxix) EENLLDFVRF from EBV (SEQ ID NO: 120);
(lxx) KEHVIQNAF from EBV (SEQ ID NO: 121);
(lxxi) RRIYDLIEL from EBV (SEQ ID NO: 122);
(lxxii) QPRAPIRPI from EBV (SEQ ID NO: 123);
(lxxiii) EGGVGWRHW from EBV (SEQ ID NO: 124);
(lxxiv) CLGGLLTMV from EBV (SEQ ID NO: 125);
(lxxv) RRRWRRLTV from EBV (SEQ ID NO: 126);
(lxxvi) RAKFKQLL from EBV (SEQ ID NO: 127);
(lxxvii)RKCCRAKFKQLLQHYR. from EBV (SEQ ID NO: 128);
(lxxviii) YLLEMLWRL from EBV (SEQ ID NO: 129);
(lxxix) YFLEILWGL from EBV (SEQ ID NO: 130);
(lxxx) YLLEILWRL from EBV (SEQ ID NO: 131);
(lxxxi) YLQQNWWTL from EBV (SEQ ID NO: 132);
(lxxxii)LLLALLFWL from EBV (SEQ ID NO: 133);
(lxxxiii) LLVDLLWLL from EBV (SEQ ID NO: 134);
(lxxxiv) LLLIALWNL from EBV (SEQ ID NO: 135);
(lxxxv)WLLLFLAIL from EBV (SEQ ID NO: 136);
(lxxxvi) TLLVDLLWL from EBV (SEQ ID NO: 137);
(lxxxvii) LLWLLLFLA from EBV (SEQ ID NO: 138);
(lxxxviii) ILLIIALYL from EBV (SEQ ID NO: 139);
(lxxxix) VLFIFGCLL from EBV (SEQ ID NO: 140);

(xc) RLGATIWQL from EBV (SEQ ID NO: 141);
(xci) ILYFIAFAL from EBV (SEQ ID NO: 142);
(xcii) SLVIVTTFV from EBV (SEQ ID NO: 143);
(xciii) LMIIPLINV from EBV (SEQ ID NO: 144);
(xciv) TLFIGSHVV from EBV (SEQ ID NO: 145);
(xcv) LIPETVPYI from EBV (SEQ ID NO: 146);
(xcvi) VLQWASLAV from EBV (SEQ ID NO: 147);
(xcvii) QLTPHTKAV from EBV (SEQ ID NO: 148);
(xcviii)SVLGPISGHVLK from HCMV pp65 (SEQ ID NO: 149);
(xcix) FTSQYRIQGKL from HCMV pp65(SEQ ID NO: 150);
(c) FVFPTKDVALR from HCMV pp65 (SEQ ID NO: 151);
(ci) FPTKDVAL from HCMV pp65 (SEQ ID NO: 152);
(cii) NLVPMVATV from HCMV pp65 (SEQ ID NO: 153);
(ciii) MLNIPSINV from HCMV pp65 (SEQ ID NO: 154);
(civ) RIFAELEGV from HCMV pp65 (SEQ ID NO: 155);
(cv) TPRVTGGGGAM from HCMV pp65 (SEQ ID NO: 156);
(cvi) RPHERNGFTVL from HCMV pp65 (SEQ ID NO: 157);
(cvii) RLLQTGIHV from HCMV pp65 (SEQ ID NO: 158);
(cviii) VIGDQYVKV from HCMV pp65 (SEQ ID NO: 159);
(cix) ALFFFDIDL from HCMV pp65 (SEQ ID NO: 160);
(cx) YSEHPTFTSQY from HCMV pp65 (SEQ ID NO: 161);
(cxi) VLCPKNMII from HCMV pp65 (SEQ ID NO: 162);
(cxii) DIYRIFAEL from HCMV pp65 (SEQ ID NO: 163);
(cxiii) ILARNLVPMV from HCMV pp65 (SEQ ID NO: 164);
(cxiv) EFFWDANDIY from HCMV pp65 (SEQ ID NO: 165);
(cxv) IPSINVHHY) from HCMV pp65 (SEQ ID NO: 166);
(cxvi) YILEETSVM from HCMV IE-1 (SEQ ID NO: 167);
(cxvii) CVETMCNEY from HCMV IE-1 (SEQ ID NO: 168);
(cxviii)RRIEEICMK from HCMV IE-1 (SEQ ID NO: 169);
(cxix) TTVYPPSSTAK from HCMV pp150 (SEQ ID NO: 170);
(cxx) RRYPDAVYL from Measles Virus Fusion glycoprotein (SEQ ID NO: 171);
(cxxi) GYKDGNEYI from *Listeria monocytogenes* (SEQ ID NO: 172);
(cxxii) SIINFEKL from ovalbumin (SEQ ID NO: 173); and
(cxxiii) DLMGYIPLV from the core protein of hepatitis C virus (SEQ ID NO: 176).

It will be apparent from the preceding description that the polypeptide moiety of the subject lipopeptide is synthesized conveniently as a single amino acid chain, thereby requiring no post-synthesis modification to incorporate both epitopes. As exemplified herein, a polypeptide moiety comprising an amino acid sequence selected from the group consisting of the following is preferred:

(i) ALNNRFQI**K**GVEL**K**STYQRTRALV (SEQ ID NO: 3);
(ii) ALNNRFQI**K**GVEL**K**S**K**TYQRTRALV (SEQ ID NO: 4);
(iii) KLIPNASLIENCT**K**AEL**K**TYQRTRALV (SEQ ID NO: 5);
(iv) KLIPNASLIENCT**K**AEL**K**NLVPMVATV (SEQ ID NO: 6);
(v) AELGEYE**K**LLNSVLEPI**K**NLVPMVATV (SEQ ID NO: 7);
(vi) TAAQITAGIALHQSNLN**K**NLVPMVATV (SEQ ID NO: 8);
(vii) PRYIATNGYLISNFDES**K**NLVPMVATV (SEQ ID NO: 9);
(viii) KLIPNASLIENCT**K**AEL**K**YLLEMLWRL (SEQ ID NO: 10);
(ix) AELGEYE**K**LLNSVLEPI**K**YLLEMLWRL (SEQ ID NO: 11);
(x) TAAQITAGIALHQSNLN**K**YLLEMLWRL (SEQ ID NO: 12);
(xi) PRYIATNGYLISNFDES**K**YLLEMLWRL (SEQ ID NO: 13);
(xii) KLIPNASLIENCT**K**AEL**K**SIINFE**K**L (SEQ ID NO: 174);
(xiii) KLIPNASLIENCT**K**AEL**K**GYKDGNEYI (SEQ ID NO: 175) and
(xiv) KLIPNASLIENCT**K**AEL**K**DLMGYIPLV (SEQ ID NO: 177).

For the purposes of nomenclature, SEQ ID Nos: 3-4 relate to synthetic peptides comprising a T-helper epitope from the light chain of influenza virus hemagglutinin (i.e. SEQ ID NO: 1) and an immunodominant *H-2*^{d}-restricted CTL epitope from the nucleoprotein of influenza virus strain PR8 (i.e. SEQ ID NO: 2) wherein the internal lysine residue that provides a lipid attachment site at its epsilon-amino group is indicated in bold type. In SEQ ID No: 4, an additional internal lysine residue has been engineered between the T-helper and CTL epitope (K16 in SEQ ID NO: 4).

SEQ ID No: 5 relates to a synthetic peptide comprising a T-helper epitope from canine distemper virus (CDV-F; SEQ ID NO: 20) that is active in dogs, mice, and humans and an immunodominant *H-2*^{d}-restricted CTL epitope from the

nucleoprotein of influenza virus strain PR8 (i.e. SEQ ID NO: 2) wherein the internal lysine residue that provides a lipid attachment site at its epsilon-amino group is indicated in bold type. In this peptide, an additional internal lysine residue has been engineered between the T-helper and CTL epitope (K18 in SEQ ID NO: 5).

SEQ ID No: 6 relates to a synthetic peptide comprising a T-helper epitope from canine distemper virus (CDV-F; SEQ ID NO: 20) that is active in dogs, mice, and humans and an immunodominant HLA A2-restricted CTL epitope from the immunodominant pp65 antigen of the cytomegalovirus of humans (i.e. HCMV pp65 antigen) (i.e. SEQ ID NO: 153) wherein the internal lysine residue that provides a lipid attachment site at its epsilon-amino group is indicated in bold type. In this peptide, an additional internal lysine residue has been engineered between the T-helper and CTL epitope (K18 in SEQ ID NO: 6).

SEQ ID No: 7 relates to a synthetic peptide comprising a T-helper epitope from canine distemper virus (CDV-F; SEQ ID NO: 22) that is active in dogs, mice, and humans and an immunodominant HLA A2-restricted CTL epitope from HCMV pp65 antigen (i.e. SEQ ID NO: 153) wherein the internal lysine residue that provides a lipid attachment site at its epsilon-amino group is indicated in bold type. In this peptide, an additional internal lysine residue has been engineered between the T-helper and CTL epitope (K18 in SEQ ID NO: 7).

SEQ ID No: 8 relates to a synthetic peptide comprising a T-helper epitope from canine distemper virus (CDV-F; SEQ ID NO: 16) that is active in dogs, mice, and humans and an immunodominant HLA A2-restricted CTL epitope from HCMV pp65 antigen (i.e. SEQ ID NO: 153) wherein the internal lysine residue that provides a lipid attachment site at its epsilon-amino group is indicated in bold type. In this peptide, an additional internal lysine residue has been engineered between the T-helper and CTL epitope (K18 in SEQ ID NO: 8).

SEQ ID No: 9 relates to a synthetic peptide comprising a T-helper epitope from canine distemper virus (CDV-F; SEQ ID NO: 40) that is active in dogs, mice, and humans and an immunodominant HLA A2-restricted CTL epitope from HCMV

pp65 antigen (i.e. SEQ ID NO: 153) wherein the internal lysine residue that provides a lipid attachment site at its epsilon-amino group is indicated in bold type. In this peptide, an additional internal lysine residue has been engineered between the T-helper and CTL epitope (K18 in SEQ ID NO: 9).

SEQ ID No: 10 relates to a synthetic peptide comprising a T-helper epitope from canine distemper virus (CDV-F; SEQ ID NO: 20) that is active in dogs, mice, and humans and an immunodominant HLA A2-restricted CTL epitope from Epstein-Barr virus LMP1 antigen (i.e. EBV LMP1; SEQ ID NO: 129) wherein the internal lysine residue that provides a lipid attachment site at its epsilon-amino group is indicated in bold type. In this peptide, an additional internal lysine residue has been engineered between the T-helper and CTL epitope (K18 in SEQ ID NO: 10).

SEQ ID No: 11 relates to a synthetic peptide comprising a T-helper epitope from canine distemper virus (CDV-F; SEQ ID NO: 22) that is active in dogs, mice, and humans and an immunodominant HLA A2-restricted CTL epitope from EBV LMP1 (SEQ ID NO: 129) wherein the internal lysine residue that provides a lipid attachment site at its epsilon-amino group is indicated in bold type. In this peptide, an additional internal lysine residue has been engineered between the T-helper and CTL epitope (K18 in SEQ ID NO: 11).

SEQ ID No: 12 relates to a synthetic peptide comprising a T-helper epitope from canine distemper virus (CDV-F; SEQ ID NO: 16) that is active in dogs, mice, and humans and an immunodominant HLA A2-restricted CTL epitope from EBV LMP1 (SEQ ID NO: 129) wherein the internal lysine residue that provides a lipid attachment site at its epsilon-amino group is indicated in bold type. In this peptide, an additional internal lysine residue has been engineered between the T-helper and CTL epitope (K18 in SEQ ID NO: 12).

SEQ ID No: 13 relates to a synthetic peptide comprising a T-helper epitope from canine distemper virus (CDV-F; SEQ ID NO: 40) that is active in dogs, mice, and humans and an immunodominant HLA A2-restricted CTL epitope from EBV LMP1 (SEQ ID NO: 129) wherein the internal lysine residue that provides a lipid attachment site at its epsilon-amino group is indicated in bold type. In this

peptide, an additional internal lysine residue has been engineered between the T-helper and CTL epitope (K18 in SEQ ID NO: 13).

SEQ ID No: 174 relates to a synthetic peptide comprising a T-helper epitope from canine distemper virus (CDV-F; SEQ ID NO: 20) that is active in dogs, mice, and humans and an immunodominant CTL epitope from ovalbumin(i.e. SEQ ID NO: 173) wherein the internal lysine residues that provide possible lipid attachment sites at its epsilon-amino group are indicated in bold type. Preferably, the lipid is attached via K18 in SEQ ID NO: 174, which is an additional internal lysine residue that has been engineered between the T-helper and CTL epitope.

SEQ ID No: 175 relates to a synthetic peptide comprising a T-helper epitope from canine distemper virus (CDV-F; SEQ ID NO: 20) that is active in dogs, mice, and humans and an immunodominant CTL epitope from a *Listeria monocytogenes* antigen (i.e. SEQ ID NO: 172) wherein the internal lysine residues that provide possible lipid attachment sites at its epsilon-amino group are indicated in bold type. Preferably, the lipid is attached via K18 in SEQ ID NO: 175 which is an additional internal lysine residue that has been engineered between the T-helper and CTL epitope.

SEQ ID No: 177 relates to a synthetic peptide comprising a T-helper epitope from canine distemper virus (CDV-F; SEQ ID NO: 20) that is active in dogs, mice, and humans and an immunodominant CTL epitope from the core protein of hepatitis C virus (SEQ ID NO: 176) wherein the internal lysine residues that provide possible lipid attachment sites at its epsilon-amino group are indicated in bold type. Preferably, the lipid is attached via K18 in SEQ ID NO: 177.

The skilled artisan will readily be able to synthesize additional polypeptide moieties to those exemplified herein for use in the subject lipopeptides, by substituting the T-helper epitope and/or the CTL epitope of anyone of SEQ ID Nos: 3-13, 174, 175 or 177 with another T-helper epitope or CTL epitope, such as, for example a T-helper epitope set forth in any one of SEQ ID Nos: 14-52, or a CTL epitope set forth in any one of SEQ ID Nos: 53-173 or 176. Moreover, the selection of appropriate T-helper epitope and CTL combinations will be apparent

to the skilled artisan from the disclosure provided herein, according to the target species and the CTL epitope against which an immune response is sought.

The amino acid sequences of the polypeptide moities described herein, including those exemplified polypeptides set forth in SEQ ID Nos: 3-13, 174, 175 and 177 may be modified for particular purposes according to methods well known to those of skill in the art without adversely affecting their immune function. For example, particular peptide residues may be derivatized or chemically modified in order to enhance the immune response or to permit coupling of the peptide to other agents, particularly lipids. It also is possible to change particular amino acids within the peptides without disturbing the overall structure or CTL immunogenicity of the peptide. Such changes are therefore termed "conservative" changes and tend to rely on the hydrophilicity or polarity of the residue. The size and/or charge of the side chains also are relevant factors in determining which substitutions are conservative.

It is well understood by the skilled artisan that, inherent in the definition of a biologically functional equivalent protein or peptide, is the concept that there is a limit to the number of changes that may be made within a defined portion of the molecule and still result in a molecule with an acceptable level of equivalent biological activity. Biologically functional equivalent peptides are thus defined herein as those peptides in which specific amino acids may be substituted. Particular embodiments encompass variants that have one, two, three, four, five or more variations in the amino acid sequence of the peptide. Of course, a plurality of distinct proteins/peptides with different substitutions may easily be made and used in accordance with the invention.

Those skilled in the art are well aware that the following substitutions are permissible conservative substitutions (i) substitutions involving arginine, lysine and histidine; (ii) substitutions involving alanine, glycine and serine; and (iii) substitutions involving phenylalanine, tryptophan and tyrosine. Peptides incorporating such conservative substitutions are defined herein as biologically functional equivalents.

The importance of the hydropathic amino acid index in conferring interactive biological function on a protein is generally understood in the art (Kyte & Doolittle, *J. Mol. Biol. 157*, 105-132, 1982). It is known that certain amino acids may be substituted for other amino acids having a similar hydropathic index or score and still retain a similar biological activity.The hydropathic index of amino acids also may be considered in determining a conservative substitution that produces a functionally equivalent molecule. Each amino acid has been assigned a hydropathic index on the basis of their hydrophobicity and charge characteristics, as follows: isoleucine (+4.5); valine (+4.2); leucine (+3.8); phenylalanine (+2.8); cysteine/cystine (+2.5); methionine (+1.9); alanine (+1.8); glycine (-0.4); threonine (-0.7); serine (-0.8); tryptophan (-0.9); tyrosine (-1.3); proline (-1.6); histidine (-3.2); glutamate (-3.5); glutamine (-3.5); aspartate (-3.5); asparagine (-3.5); lysine (-3.9); and arginine (-4.5). In making changes based upon the hydropathic index, the substitution of amino acids whose hydropathic indices are within .+/- 0.2 is preferred. More preferably, the substitution will involve amino acids having hydropathic indices within +/- 0.1, and more preferably within about +/- 0.05.

It is also understood in the art that the substitution of like amino acids is made effectively on the basis of hydrophilicity, particularly where the biological functional equivalent protein or peptide thereby created is intended for use in immunological embodiments, as in the present case (e.g. US Patent No. 4,554,101). As detailed in US Patent No. 4,554,101, the following hydrophilicity values have been assigned to amino acid residues: arginine (+3.0); lysine (+3.0); aspartate (+3.0 +/- 0.1); glutamate (+3.0 +/- 0.1); serine (+0.3); asparagine (+0.2); glutamine (+0.2); glycine (0); threonine (-0.4); proline (-0.5 +/- 0.1); alanine (-0.5); histidine (-0.5); cysteine (-1.0); methionine (-1.3); valine (-1.5); leucine (-1.8); isoleucine (-1.8); tyrosine (-2.3); phenylalanine (-2.5); tryptophan (-3.4). In making changes based upon similar hydrophilicity values, it is preferred to substitute amino acids having hydrophilicity values within about +/- 0.2 of each other, more preferably within about +/- 0.1, and even more preferably within about +/- 0.05.

Having identified peptides suitable for use as immunogens, it also is contemplated that other sterically similar compounds may be formulated to mimic the key portions of the peptide structure. Such compounds, which may be termed

peptidomimetics, may be used in the same manner as the peptides of the invention and hence are also functional equivalents. The generation of a structural functional equivalent may be achieved by the techniques of modeling and chemical design known to those of skill in the art. It will be understood that all such sterically similar constructs fall within the scope of the present invention.

Another method for determining the "equivalence" of modified peptides involves a functional approach. For example, a suitable variant peptide will comprise an amino acid sequence that interacts at a significant level with a MHC Class I allele as determined using a predictive algorithm for determining MHC Class I-binding epitopes, such as, for example, the SYFPEITHI algorithm of the University of Tuebingen, Germany, or the algorithm of the HLA Peptide Binding Predictions program of the BioInformatics and Molecular Analysis Section (BIMAS) of the National Institutes of Health of the Government of the United States of America. Such variant sequences will also bind to and/or stabilize an MHC Class I molecule on the surface of an APC (eg in the PBMC fraction or buffy coat fraction of serum) and/or will induce a memory CTL response or elicit IFN-γ production and/or will stimulate CTL activity in a standard cytotoxicity assay. The determination of such functionalities is readily achievable by those skilled in the art.

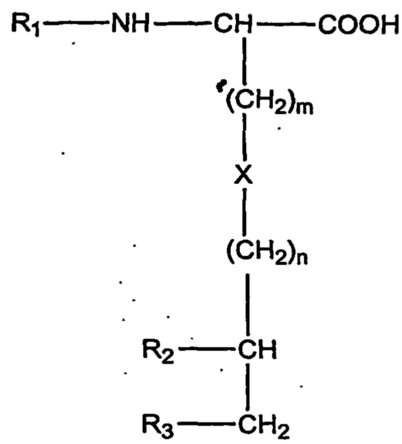
The polypeptide moiety of the lipopeptide is readily synthesized using standard techniques, such as the Merrifield method of synthesis (Merrifield, *J Am Chem Soc, 85,*:2149-2154, 1963) and the myriad of available improvements on that technology (see e.g., Synthetic Peptides: A User's Guide, Grant, ed. (1992) W.H. Freeman & Co., New York, pp. 382; Jones (1994) The Chemical Synthesis of Peptides, Clarendon Press, Oxford, pp. 230.); Barany, G. and Merrifield, R.B. (1979) in *The Peptides* (Gross, E. and Meienhofer, J. eds.), vol. 2, pp. 1-284, Academic Press, New York; Wünsch, E., ed. (1974) *Synthese von Peptiden in Houben-Weyls Methoden der Organischen Chemie* (Müler, E., ed.), vol. 15, 4th edn., Parts 1 and 2, Thieme, Stuttgart; Bodanszky, M. (1984) *Principles of Peptide Synthesis*, Springer-Verlag, Heidelberg; Bodanszky, M. & Bodanszky, A. (1984) *The Practice of Peptide Synthesis, Springer-Verlag,* Heidelberg; Bodanszky, M. (1985) *Int. J. Peptide Protein Res.* 25, 449-474.

The lipid moiety may comprise any C_2 to C_{30} saturated, monounsaturated, or polyunsaturated linear or branched fatty acyl group, and preferably a fatty acid group selected from the group consisting of: palmitoyl, myristoyl, stearoyl, lauroyl, octanoyl and decanoyl. Lipoamino acids are particularly preferred lipid moieties within the present context. As used herein, the term "lipoamino acid" refers to a molecule comprising one or two or three or more lipids covalently attached to an amino acid residue, such as, for example, cysteine or serine, lysine or an analog thereof. In a particularly preferred embodiment, the lipoamino acid comprises cysteine and optionally, one or two or more serine residues.

The structure of the lipid moiety is not essential to activity of the resulting lipopeptide and, as exemplified herein, palmitic acid and/or cholesterol and/or Pam_1Cys and/or Pam_2Cys and/or Pam_3Cys can be used. The present invention clearly contemplates a range of other lipid moieties for use in the lipopeptides, such as, for example, lauric acid, stearic acid or octanoic acid, without loss of immunogenicity. Accordingly, the present invention is not to be limited by the structure of the lipid moiety, unless specified otherwise, or the context requires otherwise.

Similarly, the present invention is not to be limited by a requirement for a single lipid moiety unless specified otherwise or the context requires otherwise. The additon of multiple lipid moieties to the peptide moiety, such as, for example, to a position within the T-helper epitope, and to a position between the T-helper epitope and the B-cell epitope, is clearly contemplated.

The lipid moiety is preferably a compound having a structure of General Formula (VII):



wherein:

(i) X is selected from the group consisting of sulfur, oxygen, disulfide (-S-S-), and methylene ($-CH_2-$), and amino (-NH-);

(ii) m is an integer being 1 or 2;

(iii) n is an integer from 0 to 5;

(iv) R_1 is selected from the group consisting of hydrogen, carbonyl (-CO-), and R'-CO- wherein R' is selected from the group consisting of alkyl having 7 to 25 carbon atoms, alkenyl having 7 to 25 carbon atoms, and alkynyl having 7 to 25 carbon atoms, wherein said alkyl, alkenyl or alkynyl group is optionally substituted by a hydroxyl, amino, oxo, acyl, or cycloalkyl group;

(v) R_2 is selected from the group consisting of R'-CO-O-, R'-O-, R'-O-CO-, R'-NH-CO-, and R'-CO-NH-, wherein R' is selected from the group consisting of alkyl having 7 to 25 carbon atoms, alkenyl having 7 to 25 carbon atoms, and alkynyl having 7 to 25 carbon atoms, wherein said alkyl, alkenyl or alkynyl group is optionally substituted by a hydroxyl, amino, oxo, acyl, or cycloalkyl group; and

(vi) R_3 is selected from the group consisting of R'-CO-O-, R'-O-, R'-O-CO-, R'-NH-CO-, and R'-CO-NH-, wherein R' is selected from the group consisting of alkyl having 7 to 25 carbon atoms, alkenyl having 7 to 25 carbon atoms, and alkynyl having 7 to 25 carbon atoms, wherein said alkyl, alkenyl or alkynyl group is optionally substituted by a hydroxyl, amino, oxo, acyl, or cycloalkyl group

and wherein each of R_1, R_2 and R_3 are the same or different.

Depending upon the substituent, the lipid moiety of general structure V may be a chiral molecule, wherein the carbon atoms directly or indirectly covalently bound to integers R_1 and R_2 are asymmetric dextrorotatory or levorotatory (i.e. an R or S) configuration.

Preferably, X is sulfur; m and n are both 1; R_1 is selected from the group consisting of hydrogen, and R'-CO-, wherein R' is an alkyl group having 7 to 25 carbon atoms; and R_2 and R_3 are selected from the group consisting of R'-CO-O-, R'-O-, R'-O-CO-, R'-NH-CO-, and R'-CO-NH-, wherein R' is an alkyl group having 7 to 25 carbon atoms.

Preferably, R' is selected from the group consisting of: palmitoyl, myristoyl, stearoyl and decanoyl. More preferably, R' is palmitoyl.

Each integer R' in said lipid moiety may be the same or different.

In a particularly preferred embodiment, X is sulfur; m and n are both 1; R_1 is hydrogen or R'-CO- wherein R' is palmitoyl; and R_2 and R_3 are each R'-CO-O- wherein R' is palmitoyl. These particularly preferred compounds are shown by Formula (I) and Formula (II) *supra*.

The lipid moiety can also have the following General Formula (VIII):

$$R_4\text{—NH—}\underset{\displaystyle R_5}{\underset{|}{\text{CH}}}\text{—COOH}$$

wherein:

(i) R_4 is selected from the group consisting of: (i) an alpha-acyl-fatty acid residue consisting of between about 7 and about 25 carbon atoms; (ii) an alpha-alkyl-beta-hydroxy-fatty acid residue; (iii) a beta-hydroxy ester of an alpha-alkyl-beta-hydroxy-fatty acid residue wherein the ester group is preferably a straight chain or branched chain comprising more than 8 carbon atoms; and (iv) a lipoamino acid residue; and

(ii) R_5 is hydrogen or the side chain of an amino acid residue.

Preferably, R_4 consists of between about 10 and about 20 carbon atoms, and more preferably between about 14 and about 18 carbon atoms.

Optionally, wherein R_4 is a lipoamino acid residue, the side-chain of the integers R_4 and R_5 can form a covalent linkage. For example, wherein R_4 comprises an amino acid selected from the group consisting of lysine, ornithine, glutamic acid, aspartic acid, a derivative of lysine, a derivative of ornithine, a derivative of glutamic acid, and a derivative of aspartic acid, then the side chain of that amino acid or derivative is covalently attached, by virtue of an amide or ester linkage, to R_5.

Preferably, the structure set forth in General Formula VIII is a lipid moiety selected from the group consisting of: N,N'-diacyllysine; N,N'-diacylornithine; di(monoalkyl)amide or ester of glutamic acid; di(monoalkyl)amide or ester of aspartic acid; a N,O-diacyl derivative of serine, homoserine, or threonine; and a N,S-diacyl derivative of cysteine or homocysteine.

Amphipathic molecules, particularly those having a hydrophobicity not exceeding the hydrophobicity of Pam_3Cys (Formula (I)) are also preferred.

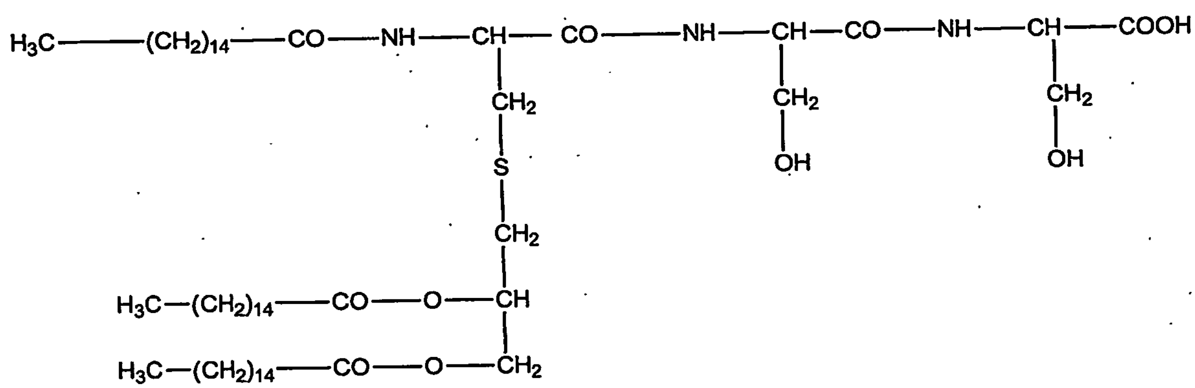
The lipid moieties of Formula (I), Formula (II), Formula (VI) or Formula (VIII) are further modified during synthesis or post-synthetically, by the addition of one or more spacer molecules, preferably a spacer that comprises carbon, and more preferably one or more amino acid residues. These are conveniently added to the lipid structure via the terminal carboxy group in a conventional condensation, addition, substitution, or oxidation reaction. The effect of such spacer molecules is to separate the lipid moiety from the polypeptide moiety and increase immunogenicity of the lipopeptide product.

Serine dimers, trimers, tetramers, etc, are particularly preferred for this purpose.

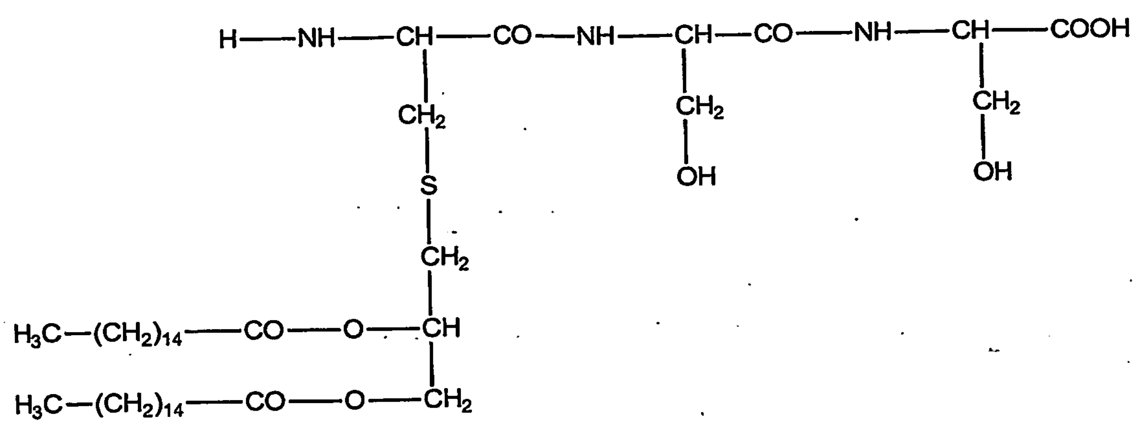
Preferably, such spacers include a terminal protected amino acid residue to facilitate the later conjugation of the modified lipoamino acid to the polypeptide.

Exemplary modified lipoamino acids produced according to this embodiment are presented as Formulae (III) and (IV), which are readily derived from Formulae (I) and (II), respectively by the addition of a serine homodimer. As exemplified herein, Pam_3Cys of Formula (I), or Pam_2Cys of Formula (II) is conveniently synthesized as the lipoamino acids Pam_3Cys-Ser-Ser of Formula (III), or Pam_2Cys-Ser-Ser of Formula (IV) for this purpose.

Formula (III):



Formula (IV):



As an alternative to the addition of a spacer to the lipid moiety, the spacer may be added to the epsilon amino group of the internal lysine residue or to the terminal side-chain group of a lysine analog in the polypeptide moiety, either as a short

peptide, such as, for example a serine homodimer, homotrimer, homotetramer, etc, or alternatively, by the sequential addition of amino acid residues, thereby producing a branched polypeptide chain. This approach takes advantage of the modified nature of the terminal side-chain group on the internal lysine or lysine analog to achieve specificity in the addition of the spacer. Naturally, to avoid sequential spacer addition, the terminal amino acid residue of the spacer should preferably be protected, such that de-protection can facilitate conjugation.of the lipid moiety to the branched polypeptide.

Alternatively, the spacer may be added to a non-modified epsilon amino group of the polypeptide by conventional nucleophilic substitution reaction. However, it is preferred to follow this approach if the polypeptide has an amino acid sequence comprising a single internal lysine residue and a blocked N-terminus.

The lipid moiety is prepared by conventional synthetic means, such as, for example, the methods described in US Patent Nos. 5,700,910 and 6,024,964, or alternatively, the method described by Wiesmuller *et al., Hoppe Seylers Zur Physiol. Chem. 364*, 593 (1983), Zeng *et al., J. Pept. Sci 2*, 66 (1996), Jones *et al., Xenobiotica 5*, 155 (1975), or Metzger *et al., Int. J. Pept.Protein Res. 38*, 545 (1991). Those skilled in the art will be readily able to modify such methods to achieve the synthesis of a desired lipid for use conjugation to a polypeptide.

Combinations of different lipids are also contemplated for use in the lipopeptides of the invention. For example, one or two myristoyl-containing lipids or lipoamino acids are attached via internal lysine or lysine analog residues to the polypeptide moiety, optionally separated from the polypeptide by a spacer, with one or two palmitoyl-containing lipid or lipoamino acid molecules attached to carboxy terminal lysine amino acid residues. Other combinations are not excluded.

The lipopeptides of the invention are readily modified for diagnostic purposes. For example, it is modified by addition of a natural or synthetic hapten, an antibiotic, hormone, steroid, nucleoside, nucleotide, nucleic acid, an enzyme, enzyme substrate, an enzyme inhibitor, biotin, avidin, polyethylene glycol, a peptidic polypeptide moiety (e.g. tuftsin, polylysine), a fluorescence marker (e.g. FITC,

RITC, dansyl, luminol or coumarin), a bioluminescence marker, a spin label, an alkaloid, biogenic amine, vitamin, toxin (e.g. digoxin, phalloidin, amanitin, tetrodotoxin), or a complex-forming agent.

As exemplified herein, highly immunogenic lipopeptides capable of inducing CTL responses are provided, said lipopeptides comprising Pam_3Cys of Formula (I), or Pam_2Cys of Formula (II) conjugated via the epsilon amino group of a lysine residue positioned between the $CD4^+$ T-helper epitope and a $CD8^+$ CTL epitope.

Preparation of lipopeptides

A second aspect of the invention provides a method of producing a lipopeptide comprising:

(i) producing a polypeptide comprising an amino acid sequence that comprises:

 (a) the amino acid sequence of a T helper cell (Th) epitope and the amino acid sequence of a CTL epitope, wherein said amino acid sequences are different; and

 (b) one or more internal lysine residues or internal lysine analog residues; and

(iii) covalently attaching each of said one or more lipid moieties directly or indirectly to an epsilon-amino group of said one or more internal lysine residues or to the terminal side-chain group of said one or more internal lysine analog residues so as to produce a lipopeptide having the lipid moiety attached to the epsilon amino group of said internal lysine residue or having the lipid moiety attached to the terminal side-chain group of said internal lysine analog residue.

Preferably, the method further comprises production of the lipid moiety.

Conventional chemical syntheses referred to herein are the preferred means for producing the polypeptide moiety and the lipid moiety.

Preferably, the internal lysine or lysine analog is modified by selective removal of a blocking group (eg: Mtt) from the terminal side-chain group so as to permit the

addition of an amino acid residue, a spacer or lipid moiety, including a lipoamino acid, at that position.

For attachment of the lipid to the polypeptide, it is convenient for the functional groups of the polypeptide to be protected in a manner known in the art of peptide synthesis, to ensure that no undesirable reactions at those groups takes place at a significant reaction rate.

By known coupling processes, the polypeptide is synthesized on a solid or soluble carrier, such as a polymer (for example Merrifield resin) and made available for conjugation to a spacer, amino acid, or lipid. For example, the terminal side chain group of the lysine or lysine analog (eg. epsilon amino group of the internal lysine) is protected by one of a number of protecting groups. Blocking groups (also called protecting groups or masking groups) are used to protect the amino group of the amino acid having an activated carboxyl group that is involved in the coupling reaction, or to protect the carboxyl group of the amino acid having an acylated amino group that is involved in the coupling reaction. For coupling to occur, a blocking group must be removed without disrupting a peptide bond, or any protecting group attached to another part of the peptide.

For solid phase peptide synthesis, blocking groups that are stable to the repeated treatments necessary for removal of the amino blocking group of the growing peptide chain and for repeated amino acid couplings, are used for protecting the amino acid side-chains. Additionally, the peptide-resin anchorage that protects the C-terminus of the peptide must be protected throughout the synthetic process until cleavage from the resin is required. Accordingly, by the judicious selection of orthogonally protected alpha-amino acids, lipids and/or amino acids are added at desired locations to a growing peptide whilst it is still attached to the resin.

Preferred amino blocking groups are easily removable but sufficiently stable to survive conditions for the coupling reaction and other manipulations, such as, for example, modifications to the side-chain groups. Preferred amino blocking groups are slected from the group consisting of: (i) a benzyloxycarbonyl group (Z or carbobenzoxy) that is removed easily by catalytic hydrogenation at room

temperature and ordinary pressure, or using sodium in liquid ammonia and hydrobromic acid in acetic acid; (ii) a t-Butoxycarbonyl group (Boc) that is introduced using t-butoxycarbonyl azide or di-tert-butyldicarbonate and removed using mild acid such as, for example, trifluoroacetic acid (50% TFA in dichloromethane), or HCl in acetic acid/dioxane/ethylacetate; (iii) a 9-fluorenylmethyloxycarbonyl group (Fmoc) that is cleaved under mildly basic, non-hydrolytic conditions, such as, for example, using a primary or secondary amine (eg. 20% piperidine in dimethyl formamide); (iv) a 2-(4-biphenylyl) propyl(2)oxycarbonyl group (Bpoc); (v) a 2-nitro-phenylsulfenyl group (Nps); and (vi) a dithia-succionyl group (Dts).

Side chain-protecting groups will vary for the functional side chains of the amino acids forming the peptide being synthesized. Side-chain protecting groups are generally based on the Bzl group or the tBu group. Amino acids having alcohols or carboxylic acids in the side-chain are protected as Bzl ethers, Bzl esters, cHex esters, tBu ethers, or tBu esters. Side-chain protection of Fmoc amino acids requires blocking groups that are ideally base stable and weak acid (TFA) labile. For example, the epsilon-amino group of Lysine is protected using Mtt (eg. Fmoc-lysine(Mtt)-OH). Alternatively, a halogenated benzyl derivative such as ClZ is used to protect the lysine side chain should enhanced acid stability be required. The thiol group of Cystine, the imidazole of Histidine, or guanidino group of Arginine, generally require specialised protection. Many different protecting groups for peptide synthesis have been described (*see* The Peptides, Gross *et al.* eds., Vol. 3, Academic Press, New York, 1981).

The two most widely used protection strategies are the Boc/Bzl- and the Fmoc/tBu-strategies. In Boc/Bzl, Boc is used for amino protection and the side-chains of the various amino acids are protected using Bzl- or cHex-based protecting groups. A Boc group is stable under catalytic hydrogenation conditions and is used orthogonally along with a Z group for protection of many side chain groups. In Fmoc/tBu, Fmoc is used for amino protection and the side-chains are protected with tBu-based protecting groups.

Peptides are lipidated by methods well known in the art. Standard condensation, addition, substitution or oxidation (e.g. disulfide bridge formation or amide bond formation between a terminal amino group on the internal lysine or lysine analog with the carboxy terminal group of an incoming amino acid or peptide or lipoamino acid) reactions result in the addition of lipid to the polypeptide.

In an alternative embodiment, a peptide of the present invention for use as an immunogen is produced by chemoselective ligation or chemical conjugation or oxime chemistry. Such methods are well-known in the art, and allow for the individual peptide components to be produced by chemical or recombinant means, followed by their chemoselective ligation in an appropriate configuration or conformation or order (eg. Nardin *et al., Vaccine 16, 590 (1998);* Nardin *et al.*, J Immunol. 166, 481 (2001); Rose *et al., Mol. Immunol. 32*, 1031 (1995); Rose *et al., Bioconjug. Chem 7,* 552 (1996); and Zeng *et al., Vaccine 18*, 1031 (2000), which are incorporated herein by reference).

Lipopeptide formulations

The lipopeptide is coveniently formulated in a pharmaceutically acceptable excipient or diluent, such as, for example, an aqueous solvent, non-aqueous solvent, non-toxic excipient, such as a salt, preservative, buffer and the like. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oil and injectable organic esters such as ethyloleate. Aqueous solvents include water, alcoholic/aqueous solutions, saline solutions, parenteral vehicles such as sodium chloride, Ringer's dextrose, etc. Preservatives include antimicrobial, anti-oxidants, chelating agents and inert gases. The pH and exact concentration of the various components the pharmaceutical composition are adjusted according to routine skills in the art.

The addition of an extrinsic adjuvant to the lipopeptide formulation, although generally not required, is also encompassed by the invention. Such extrinsic adjuvants include all acceptable immunostimulatory compounds such as, for example, a cytokine, toxin, or synthetic composition. Exemplary adjuvants include IL-1, IL-2, BCG, aluminum hydroxide, N-acetyl-muramyl-L-threonyl-D-isoglutamine (thur-MDP), N-acetyl-nor-muramyl-L-alanyl-D-isoglutamine (CGP

11637, referred to as nor-MDP), N-acetylmuramyl-L-alanyl-D-isoglutaminyl-L-alanine-2-(1'-2'-dipalmitoyl-sn-glycero-3-hydroxyphosphoryloxy)-ethylamine (CGP) 1983A, referred to as MTP-PE), lipid A, MPL and RIBI, which contains three components extracted from bacteria, monophosphoryl lipid A, trehalose dimycolate and cell wall skeleton (MPL+TDM+CWS) in a 2% squalene/Tween 80 emulsion.

It may be desirable to co-administer biologic response modifiers (BRM) with the lipopeptide, to down regulate suppressor T cell activity. Exemplary BRM's include, but are not limited to, Cimetidine (CIM; 1200 mg/d) (Smith/Kline, PA, USA); Indomethacin (IND; 150 mg/d) (Lederle, NJ, USA); or low-dose Cyclophosphamide (CYP; 75, 150 or 300 mg/m.sup.2) (Johnson/Mead, NJ, USA).

Use of the lipopeptide in immunization

The novel lipopeptides of the invention differ in essential aspects from known lipopeptide conjugates of CTL epitopes in having the lipid moiety conjugated exclusively through the terminal side-chain group of an internal lysine or lysine analog residue, thereby enhancing T cell responses without the administration of additional adjuvant. Accordingly, a particular utility of the lipopeptides of the present invention is in the fields of eliciting a T cell response either *in vivo* or *ex vivo*, synthetic vaccine preparation, diagnostic methods employing T cells, and immunotherapy for veterinary and human medicine.

More particularly, the lipopeptide of the present invention enhances CTL memory responses against the CTL epitope moiety when administered to an animal subject, without any requirement for an adjuvant to achieve a similar level of CTL activation. In addition, enhanced maturation of dendritic cells and other biological effects which include induction of IFN-γ producing CD8+ cells as well as viral, bacterial and tumour cell clearance have been observed following administration of vaccine.

Accordingly, a further aspect of the invention provides a method of enhancing cell mediated immunity against the organism from which the CTL epitope is derived in

a subject comprising administering the lipopeptide of the invention or a derivative or a functionally equivalent variant of said lipopeptide or a vaccine composition comprising said lipopeptide or variant or derivative for a time and under conditions sufficient to activate a CTL and/or a CTL precursor of said subject.

By "CTL precursor" is meant a naive T cell (ie. a T cell that expresses one or more T cell receptors on its surface and is capable of proliferating and differentiating into a memory T cell or effector T cell).

Preferably, the lipopeptide or vaccine is administered prophylactically to a subject not harboring a latent or active infection by a parasite, bacterium or virus or suffering from a cancer or administered therapeutically to a subject harboring a latent or active infection by a parasite, bacteria or virus or suffering from a cancer. In the present context, the term "activate" means that the ability of a T cell to recognize and lyse a cell harboring an antigen from which the CTL epitope is derived is enhanced, or that the ability of a T cell to recognize a T cell epitope of said antigen is enhanced, either transiently or in a sustained manner. The term "activate" shall also be taken to include a reactivation of a T cell population following activation of a latent infection by a parasite or bacteria or virus, or following re-infection with a parasite or bacteria or virus, or following immunization of a previously-infected subject with a lipopeptide or composition of the invention.

Those skilled in the art are aware that optimum T cell activation requires cognate recognition of antigen/MHC by the T cell receptor (TcR), and a co-stimulation involving the ligation of a variety of cell surface molecules on the T cell with those on an antigen presenting cell (APC). The costimulatory interactions CD28/B7, CD40L/CD40 and OX40/OX40L are preferred, but not essential for T cell activation. Other costimulation pathways may operate.

For determining the activation of a CTL or precursor CTL or the level of epitope-specific activity, standard methods for assaying the number of $CD8^+$ T cells in a specimen can be used. Preferred assay formats include a cytotoxicity assay, such as for example the standard chromium release assay, the assay for IFN-γ

production, such as, for example, the ELISPOT assay. These assay formats are described in detail in the accompanying examples.

MHC class 1 Tetramer assays can also be utilized, particularly for CTL epitope-specific quantitation of $CD8^+$ T cells (Altman *et al., Science 274*, 94-96, 1996; Ogg *et al., Curr Opin Immunol. 10,* 393-396, 1998). To produce tetramers, the carboxyl terminus of an MHC molecule, such as, for example, the HLA A2 heavy chain, is associated with a specific peptide epitope or polyepitope, and treated so as to form a tetramer complex having bound thereto a suitable reporter molecule, preferably a fluorochrome such as, for example, fluoroscein isothiocyanate (FITC), phycoerythrin, phycocyanin or allophycocyanin. Tetramer formation is achieved, for example, by producing the MHC-peptide fusion protein as a biotinylated molecule and then mixing the biotinylated MHC-peptide with deglycosylated avidin that has been labeled with a fluorophore, at a molar ratio of 4:1. The Tetramers produced bind to a distinct set of $CD8^+$ T cell receptors (TcRs) on a subset of $CD8^+$ T cells derived from the subject (eg in whole blood or a PBMC sample), to which the peptide is HLA restricted. There is no requirement for *in vitro* T cell activation or expansion. Following binding, and washing of the T cells to remove unbound or non-specifically bound Tetramer, the number of $CD8^+$ cells binding specifically to the HLA-peptide Tetramer is readily quantified by standard flow cytometry methods, such as, for example, using a FACSCalibur Flow cytometer (Becton Dickinson). The Tetramers can also be attached to paramagnetic particles or magnetic beads to facilitate removal of non-specifically bound reporter and cell sorting. Such particles are readily available from commercial sources (eg. Beckman Coulter, Inc., San Diego, CA, USA) Tetramer staining does not kill the labeled cells; therefore cell integrity is maintained for further analysis. MHC Tetramers enable the accurate quantitative analyses of specific cellular immune responses, even for extremely rare events that occur at less than 1% of $CD8^+$T cells (Bodinier *et al., Nature Med. 6,* 707-710, 2000; Ogg *et al., Curr Opin Immunol. 10,* 393-396, 1998).

The total number of $CD8^+$ cells in a sample can also be determined readily, such as, for example, by incubating the sample with a monoclonal antibody against CD8 conjugated to a different reporter molecule to that used for detecting the

Tetramer. Such antibodies are readily available (eg. Becton Dickinson). The relative intensities of the signals from the two reporter molecules used allows quantification of both the total number of $CD8^+$ cells and Tetramer-bound T cells and a determination of the proportion of total T cells bound to the Tetramer.

Because $CD4^+$ T-helper cells function in CMI as producers of cytokines, such as, for example IL-2, to facilitate the expansion of $CD8^+$ T cells or to interact with the APC thereby rendering it more competent to activate $CD8^+$ T cells, cytokine production is an indirect measure of T cell activation. Accordingly, cytokine assays can also be used to determine the activation of a CTL or precursor CTL or the level of cell mediated immunity in a human subject. In such assays, a cytokine such as, for example, IL-2, is detected or production of a cytokine is determined as an indicator of the level of epitope-specific reactive T cells.

Preferably, the cytokine assay format used for determining the level of a cytokine or cytokine production is essentially as described by Petrovsky and Harrison, *J. Immunol. Methods 186*, 37-46, 1995, which assay reference is incorporated herein.

Preferably, the cytokine assay is performed on whole blood or PBMC or buffy coat.

Preferably, the lipopeptide or derivative or variant or vaccine composition is administered for a time and under conditions sufficient to elicit or enhance the expansion of $CD8^+$ T cells.

Still more preferably, the lipopeptide or derivative or variant or vaccine composition is administered for a time and under conditions sufficient for cell mediated immunity (CMI) to be enhanced in the subject.

By "CMI" is meant that the activated and clonally expanded CTLs are MHC-restricted and specific for a CTL epitope. CTLs are classified based on antigen specificity and MHC restriction, (ie., non-specific CTLs and antigen-specific, MHC-restricted CTLs). Non-specific CTLs are composed of various cell types, including

NK cells and can function very early in the immune response to decrease pathogen load, while antigen-specific responses are still being established. In contrast, MHC-restricted CTLs achieve optimal activity later than non-specific CTL, generally before antibody production. Antigen-specific CTLs inhibit or reduce the spread of a pathogen and preferably terminate infection.

CTL activation, clonal expansion, or CMI can be induced systemically or compartmentally localized. In the case of compartmentally localized effects, it is preferred to utilize a vaccine composition suitably formulated for administration to that compartment. On the other hand, there are no such stringent requirements for inducing CTL activation, expansion or CMI systemically in the subject.

The effective amount of lipopeptide to be administered, either *solus* or in a vaccine composition to elicit CTL activation, clonal expansion or CMI will vary, depending upon the nature of the immunogenic epitope, the route of administration, the weight, age, sex, or general health of the subject immunized, and the nature of the CTL response sought. All such variables are empirically determined by art-recognized means.

The lipopeptide, optionally formulated with any suitable or desired carrier, adjuvant, BRM, or pharmaceutically acceptable excipient, is conveniently administered in the form of an injectable composition. Injection may be intranasal, intramuscular, sub-cutaneous, intravenous, intradermal, intraperitoneal, or by other known route. For intravenous injection, it is desirable to include one or more fluid and nutrient replenishers.

The optimum dose to be administered and the preferred route for administration are established using animal models, such as, for example, by injecting a mouse, rat, rabbit, guinea pig, dog, horse, cow, goat or pig, with a formulation comprising the lipopeptide, and then monitoring the CTL immune response using any conventional assay.

The use of HLA A2/K^b transgenic mice carrying a chimeric human-mouse Class I major histocompatibility complex (MHC) locus composed of the $\alpha1$ and $\alpha2$

domains of the human HLA A*0201 allele and the α3 domain of the mouse H-2K^b Class I molecules (Vitiello *et al., J. Exp. Med. 173,* 1007, 1991) is particularly preferred for testing responses *in vivo* to a lipopeptide of the invention that comprises a HLA A2-restricted CTL epitope or a vaccine composition comprising same.

Without being bound by any theory or mode of action, we believe that the biological effects of the lipopeptides are exerted through their ability to stimulate and mature dendritic cells. It is the dendritic cells which then activate CD4+ and CD8+ T cells in the draining lymph nodes. For this reason, we would not nor would it be possible to activate T cells directly as envisaged. The following section has therefore been modified accordingly to accommodate the notion of dendritic cell activation.

In a related embodiment, the invention provides a method of enhancing the cell mediated immunity of a subject, said method comprising contacting *ex vivo* cells, preferably dendritic cells, obtained from a subject with an immunologically active lipopeptide of the invention or a derivative or variant thereof or a vaccine composition comprising said lipopeptide or derivative or variant for a time and under conditions sufficient to mature said dendritic cells. Said dendritic cells are then capable of conferring epitope specific activation of T cells.

In a preferred embodiment, the invention provides a method of enhancing the cell mediated immunity of a subject, said method comprising:

(i) contacting *ex vivo* dendritic cells obtained from a subject with an immunologically active lipopeptide of the invention or a derivative or variant thereof or a vaccine composition comprising said lipopeptide or derivative or variant for a time and under conditions sufficient to mature said dendritic cells; and

(ii) introducing the activated dendritic cells autologously to the subject or syngeneically to another subject in order that T cell activation occurs.

The T cell may be a CTL or CTL precursor cell.

The subject from whom the dendritic cells are obtained may be the same subject or a different subject to the subject being treated. The subject being treated can be any subject carrying a latent or active infection by a pathogen, such as, for example, a parasite, bacterium or virus or a subject who is otherwise in need of obtaining vaccination against such a pathogen or desirous of obtaining such vaccination. The subject being treated may also be treated for a tumour that they are carrying.

By "epitope specific activity" is meant that the T cell is rendered capable of being activated as defined herein above (ie. the T cell will recognize and lyze a cell harboring a pathogen from which the CTL epitope is derived, or is able to recognize a T cell epitope of an antigen of a pathogen either transiently or in a sustained manner). Accordingly, it is particularly preferred for the T cell to be a CTL precursor which by the process of the invention is rendered able to recognize and lyze a cell harboring the pathogen or able to recognize a T cell epitope of an antigen of the pathogen either transiently or in a sustained manner.

For such an *ex vivo* application, the dendritic cells are preferably contained in a biological sample obtained from a subject, such as, for example, blood, PBMC or a buffy coat fraction derived therefrom.

Another aspect of the invention provides a method of providing or enhancing immunity against a pathogen in an uninfected subject comprising administering to said subject an immunologically active lipopeptide of the invention or a derivative or variant thereof or a vaccine composition comprising said lipopeptide or derivative or variant for a time and under conditions sufficient to provide immunological memory against a future infection by the pathogen. As with the other embodiments described herein, the pathogen may be a parasite, virus or bacterium, and is preferably a parasite, virus or bacterium referred to herein above from which a CTL epitope has been identified.

In a related embodiment, the invention provides a method of enhancing or conferring immunity against a pathogen in an uninfected subject comprising contacting *ex vivo* dendritic cells obtained from said subject with an

immunologically active lipopeptide of the invention or a derivative or variant thereof or a vaccine composition comprising said lipopeptide or derivative or variant for a time and under conditions sufficient to confer epitope specific activity on T cells.

Accordingly, this aspect of the invention provides for the administration of a prophylactic vaccine to the subject, wherein the active substituent of said vaccine (i.e. the lipopeptide of the invention) induces immunological memory via memory T cells in an uninfected individual. The preferred embodiments of vaccination protocols described herein for enhancing the cell mediated immunity of a subject apply *mutatis mutandis* to the induction of immunological memory against the pathogen in a subject.

The present invention is further described with reference to the following non-limiting examples and the drawings. The examples provided herein in mice are accepted models for equivalent diseases in humans and the skilled person will readily be capable of extending the findings presented herein for such models to a human disease context without undue experimentation.

EXAMPLE 1

Materials and Methods

Chemicals

Unless otherwise stated chemicals were of analytical grade or its equivalent. N,N'-dimethylformamide (DMF), piperidine, trifluoroacetic acid (TFA), O'benzotriazole-N,N,N',N'-tetramethyluronium hexafluorophosphate (HBTU), 1-hydroxybenzotriazole (HOBt) and diisopropylethylamine (DIPEA) and diisopropylcarbodiimide (DIPCDI) were obtained from Auspep Pty. Ltd., Melbourne, Australia and Sigma-Aldrich Pty. Ltd., Castle Hill, Australia. O'benzotriazole-N,N,N',N'-tetramethyluronium tetrafluoroborate (TBTU) was obtained from Bachem, (Bachem AG, Switzerland). Dichloromethane (DCM) and diethylether were from Merck Pty Ltd. (Kilsyth, Australia). Phenol and triisopropylsilane (TIPS) were from Aldrich (Milwaulke, WI) and trinitrobenzylsulphonic acid (TNBSA) and diaminopyridine (DMAP) from Fluka;

1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) was obtained from Sigma and palmitic acid was from Fluka.

Viruses.

The type A influenza viruses used in this study were an H3N1 subtype virus referred to as Mem 71, which was derived by genetic reassortment of A/Memphis/I/71 (H3N2) X A/Bellamy/42 (HIN1). Virus was grown for 2 days in the allantoic cavity of 10-day embryonated hen's eggs. Allantoic fluid containing virus was stored in aliquots at -70°C. Infectious virus titers were obtained by assay of plaque formation in monolayers of Madin-Darby canine kidney (MDCK) cells (Tannock *et al*, *Infect. Immun. 43,* 457-462, 1984) and are expressed as PFU/milliliter.

Bacteria

Listeria monocytogenes EGD was cultured overnight at 37°C on Horse Blood Agar (HBA) plates. The bacteria were washed off the plates using sterile PBS and the concentration adjusted to 5 x 10^3 *Listeria* cells/ml. Balb/c mice were infected intravenously with 1 x 10^3 *Listeria* cells. The dose was checked retrospectively by plating serial 10-fold dilutions on HBA plates.

Peptide Syntheses

<u>Peptides comprising influenza virus CTL epitopes</u>

A panel of immunogens was synthesized that incorporated peptides representing a minimal determinant for CD8+ T cells and/or a determinant for CD4+ T cells, both from influenza virus. The peptide NP (147-155) with the sequence TYQRTRALV (a CTL determinant present in the NP of PR8 virus; SEQ ID NO: 2) is the dominant CD8+ T-cell determinant recognized by BALB/c mice and is common to all type A influenza virus strains (Bodmer *et al*, *Cell 52*, 253-258, 1988; and Sherman *et al*, *J. Exp. Med. 175,* 1221-1226, 1992). The peptide HA2 (166-180), with the sequence ALNNRFQIKGVELKS (SEQ ID NO: 1), is a $CD4^+$ T-helper determinant present within the HA2 chain of Mem 71 influenza virus hemmagglutinin elicits CD4+ T cells that are crossreactive with all viruses of the H3 subtype (Jackson *et al*, *Virology 198*, 153-170, 1994).

Peptides comprising *L. monocytogenes* CTL epitopes

An immunogenic peptide was synthesized that incorporated a minimal CTL epitope with amino acid sequence GYKDGNEYI (residues 91-99 of the protein literialysin) from *L. monocytogenes* (ie. SEQ ID NO: 172) and a T-helper epitope from CDV-F (SEQ ID NO: 20).

Peptides comprising a CTL epitope expressed by B16-OVA tumour cell line.

An immunogenic peptide was synthesized that incorporated a CTL epitope with amino acid sequence SIINFEKL (SEQ ID NO: 173) and a T-helper epitope from CDV-F (SEQ ID NO: 20).

Peptides comprising a CTL epitope from the core protein of hepatitis C virus.

An immunogenic peptide was synthesized that incorporated a CTL epitope with amino acid sequence DLMGYIPLV (SEQ ID NO: 176) and a T-helper epitope from CDV-F (SEQ ID NO: 20).

General procedures

Synthetic immunogens were assembled by conventional solid-phase methodology using Fmoc chemistry. The general procedure used for the peptide synthesis has been described by Jackson *et al., Vaccine 18,* 355 (1999). To enable lipid attachment between the $CD4^+$ T helper epitope and the CTL epitope, Fmoc-lysine(Mtt)-OH was inserted at a point between the two epitopes in the approximate centre of the resin-bound peptide. Following completion of peptide synthesis the Mtt group was removed by continual flow washing with 1% TFA in dichloromethane over a period of 30-45 mins.

Synthesis of lipid moieties of Formulae (I)

Pam_3Cys was prepared according to the method described by Weismuller *et al., Hoppe Seylers Z Physiol Chem 364*, 593 (1983), as modified according to the method described by Zeng *et al., J Pept Sci 2,* :66 (1996). The lipoamino acid Pam_3Cys is coupled to the exposed epsilon-amino group of lysine according to

the procedure described by Zeng *et al.* (*supra*). Briefly, a 2-fold excess of Pam_3Cys, TBTU and HOBt was dissolved in DCM and a 3-fold excess of DIPEA added. This solution was then added to the resin-bound peptide to generate the lipopeptide.

Synthesis of lipid moieties of Formulae (II)

The synthesis of Pam_2Cys was adapted from previously described methods as described by Jones *et al., Xenobiotica 5, 155 (1975) and* Metzger *et al., Int J Pept Protein Res 38,* 545 (1991), with the exception that 3-bromo-propan-1,2-diol was used instead of 3-chloro-propan-1,2-diol, and centrifugation and not filtration was used to recover the product.

Synthesis of lipopeptides

Lipopeptides produced in this study had the general structures shown in Figure 1. Amino acid sequences of the peptide moieties included in the various lipopeptides are shown in Figure 2. Pam_2Cys was coupled to peptides according to the methods described by Jones *et al., Xenobiotica 5*, 155 (1975) and Metzger *et al., Int J Pept Protein Res 38*, 545 (1991), with the following modifications:

I. <u>Synthesis of S-(2,3-Dihydroxypropyl)cysteine:</u>

Triethylamine (6 g, 8.2 ml, 58 mmoles) was added to L-cysteine hydrochloride (3 g, 19 mmole) and 3-bromo-propan-1,2-diol (4.2 g, 2.36 ml, 27 mmole) in water and the homogeneous solution kept at room temperature for 3 days. The solution was reduced *in vacuo* at 40°C to a white residue which was boiled with methanol (100ml), centrifuged and the residue dissolved in water (5ml). This aqueous solution was added to acetone (300ml) and the precipitate isolated by centrifugation. The precipitate was purified by several precipitations from water with acetone to give S-(2,3-dihydroxypropyl)cysteine as a white amorphous powder (2.4 g, 12.3 mmol, 64.7%).

II. Synthesis of N-Fluorenylmethoxycarbonyl-S-(2,3-dihydroxypropyl)-cysteine (Fmoc-Dhc-OH):

S-(2,3-dihydroxypropyl)cysteine (2.45 g, 12.6 mmole) was dissolved in 9% sodium carbonate (20 ml). A solution of fluorenylmethoxycarbonyl-N-hydroxysuccinimide (3.45 g, 10.5 mmole) in acetonitrile (20 ml) was added and the mixture stirred for 2 h, then diluted with water (240 ml), and extracted with diethyl ether (25 ml x 3). The aqueous phase was acidified to pH 2 with concentrated hydrochloric acid and was then extracted with ethyl acetate (70 ml x 3). The extract was washed with water (50 ml x 2) and saturated sodium chloride solution (50 ml x 2), dried over sodium sulfate and evaporated to dryness. Recrystalisation from ether and ethyl acetate at -20°C yielded a colourless powder (2.8 g, 6.7 mmole, 63.8%).

III. Coupling of Fmoc-Dhc-OH to resin-bound peptide:

Fmoc-Dhc-OH (100mg, 0.24 mmole) was activated in DCM and DMF (1:1, v/v, 3 ml) with HOBt (36 mg, 0.24 mmole) and DICI (37 ul, 0.24 mmol) at 0 °C for 5 min. The mixture was then added to a vessel containing the resin-bound peptide (0.04 mmole, 0.25g amino-peptide resin). After shaking for 2 h the solution was removed by filtration and the resin was washed with DCM and DMF (3 x 30 ml each). The reaction was monitored for completion using the TNBSA test. If necessary a double coupling was performed.

IV. Palmitoylation of the two hydroxy groups of the Fmoc-Dhc-peptide resin:

Palmitic acid (204 mg, 0.8 mmole), DICI (154 ul, 1 mmole) and DMAP (9.76 mg, 0.08 mmole) were dissolved in 2 ml of DCM and 1 ml of DMF. The resin-bound Fmoc-Dhc-peptide resin (0.04 mmole, 0.25 g) was suspended in this solution and shaken for 16 h at room temperature. The solution was removed by filtration and the resin was then washed with DCM and DMF thoroughly to remove any residue of urea. The removal of the Fmoc group was accomplished with 2.5% DBU (2 x 5mins).

All resin-bound peptide constructs were cleaved from the solid phase support with reagent B (88% TFA, 5% phenol, 2% TIPS, 5% water) for 2 hr, and purified by reversed phase chromatography as described by Zeng *et al., Vaccine 18,* 1031 (2000).

Analytical reversed phase high pressure liquid chromatography (RP-HPLC) was carried out using a Vydac C4 column (4.6 x 300 mm) installed in a Waters® HPLC system and developed at a flow rate of 1ml/min using 0.1% TFA in H_2O and 0.1% TFA in CH_3CN as the limit solvent. All products presented as a single major peak on analytical RP-HPLC and had the expected mass when analysed by MALDI-TOF mass spectrometry on a Bruker BIFLEX instrument equipped with delayed ion extraction.

In some cases two serine residues (Ser-Ser) were added between the peptide and lipid moiety in which case serine residues were added to the ε-amino group of the central lysine residue before the lipid moiety was attached.

A schematic diagram of the peptides and lipopeptides used in this study is shown in Figure 1.

Immunization protocols

Peptides comprising influenza virus CTL epitopes

Groups of female BALB/c mice, 6 to 8 weeks old, were inoculated at day 0 and again on day 28. For subcutaneous (s.c.) inoculations 9nmoles of lipopeptide constructs were prepared in 100 µl volume of saline per dose and non-lipidated peptides formulated as an emulsion in an equal volume of complete Freund's adjuvant (CFA) for the primary injection or incomplete Freund's adjuvant for the secondary inoculation. For intranasal (i.n.) inoculations, 9nmoles of peptide in 50 µl of saline were applied to the nares of mice anaesthetised with penthrane for inhalation.

Peptides comprising a CTL epitope of *L. monocytogenes*

5 BALB/c mice were inoculated with 9nmoles of non-lipidated peptide ([P25]-*Lys*-[LLO91-99]), or lipidated peptide ([P25]-*Lys*(Pam_2Cys-Ser-Ser)-[LLO91-99]) in which lipid was attached between the two epitopes at the approximate centre of the molecule, or with 1000 bacteria. In the case of peptide vaccine, inoculation was subcutaneous and in the case of bacteria inoculation was intravenous. The number of interferon-γ producing cells present in spleen was measured on day 28

following *in vitro* stimulation with the CTL epitope or no antigen. The vertical axis shows the number of interferon-γ producing cells per 1,000,000 splenocytes.

Peptides comprising a CTL epitope of ovalbumin

Each of 9 C57BL/6 mice (8-10 wks) were immunised subcutaneously with 20 nmoles of lipidated [P25]-*Lys*(Pam_2Cys-Ser-Ser)-[SIINFEKL] or non-lipidated [P25]-*Lys*-[SIINFEKL] peptide in 100 µl volume of saline. In the case of lipidated peptide, lipid was attached between the two epitopes at the approximate centre of the molecule.

Peptides comprising a CTL epitope of hepatitis C virus core protein

Human monocyte-derived dendritic cells were incubated with lipopeptide [P25]-*Lys*(Pam_2Cys-Ser-Ser)-[HCV] (5µg/mL) for 48 hours before staining with FITC-conjugated antibodies for HLA-DR, CD83 and CD86 before analysis by flow cytometry.

Challenge of immunized mice with influenza virus

Penthrane anesthetized mice previously immunized with peptides comprising CTL epitopes of influenza virus were challenged intranasally (i.n.) with $10^{4.5}$ PFU of infectious Mem 71 influenza virus. Each mouse received 50 µl of virus in the form of allantoic fluid diluted in PBS. At 5 days after challenge, the mice were killed by cervical dislocation, and the lungs were removed and transferred aseptically to bottles containing 1.5 ml of Hank's balanced salt solution supplemented with 100 U of penicillin, 100 µg of streptomycin, and 30 µg of gentamicin per ml. Lung homogenates were prepared by using a tissue homogenizer, and the cell material was pelleted by centrifugation at 300 X *g* for 5 min. The supernatants were removed, divided into aliquots and stored at –70°C until required. Titers of infectious virus in the lung supernantants were determined by plaque assay on monolayers of MDCK cells (Tannock *et al*, *Infect. Immun. 43l,* 457-462, 1984).

Challenge of immunized mice with L. monocytogenes

Mice immunized s.c. with 9nmol peptide immunogen or PBS, or i.v. with 1000 bacteria, were challenged by i.v. injection with bacteria 28 days after priming and

the number of colony forming units of bacteria present in the liver determined 28 days after challenge.

Challenge of immunised mice with tumour cells.

Melanoma Challenge.

14 days after inoculation with non-lipidated [P25]-*Lys*-[SIINFEKL] or lipidated peptide [P25]-*Lys*(Pam_2Cys-Ser-Ser)-[SIINFEKL], 6 mice from each group were challenged with 2×10^5 melanoma cells expressing ovalbumin [B16-OVA] and therefore expressed the CTL epitope SIINFEKL (Bellone, *et al, J. Immunol. 165:*2651–2656). Hair around the injection site was removed with an electric shaver prior to injection to facilitate measurement of the emerging tumors. Growing tumors were monitored, and the animals were sacrificed when tumor size reached 15 by 15 mm. Mean tumor area was calculated for each treatment group at the indicated number of days after the tumor challenge.

Lewis Lung Carcinoma Challenge.

Mice were injected with 3×10^4 Lewis Lung tumour cells that had been transfected with ovalbumin and therefore expressed the CTL epitope (Nelson *et al., J. Immunol. 166*: 5557-5566, 2001). Four days after receiving tumour cells, animals were inoculated with 20 nmoles of lipidated peptide [P25]-*Lys*(Pam_2Cys-Ser-Ser)-[SIINFEKL], non-lipidated peptide [P25]-*Lys*-[SIINFEKL] or with PBS subcutaneously in the base of the tail. A second dose of immunogen was administered eleven days after receiving the tumour cells. Animals were monitored for tumour incidence and survival; animals were euthanased when tumour area exceeded 100 mm^2.

Tetramer staining of peptide-specific CD8+ T cells

$CD8^+$ T cells specific for an immunodominant H-$2K^d$-restricted CTL epitope consisting of amino acid residues 147-155 of the nucleoprotein of influenza virus strain A/Puerto Rico/8/34 (PR8;H1N1) in the lipopeptide immunogen, as set forth in SEQ ID NO: 2, were identified using tetrameric complexes of the H-$2K^d$ glycoprotein with bound CTL peptide (TYQRTRALV; SEQ ID NO: 2) (Bodmer *et al, Cell 52:* 253-258, 1988; Sherman *et al, J. Exp. Med. 175:* 1221-1226, 1992).

The monomer was a gift from Professor Peter Doherty, Department of Microbiology and Immunology, University of Melbourne and was made at St. Jude Children's Research Hospital, Memphis TN, USA. Tetramer was made by incubating the monomer with Streptavidin-phycoerythrin (Molecular Probes, Eugene, OR, USA) at a 4:1 molar ratio.

Lymphocytes from the lung were first treated with 20 μL of normal mouse serum (NMS) for 5 mins at room temperature and then stained for 60 min with the tetrameric complexes at a 1:25 dilution. This was followed by staining with anti-CD8α (53-6.7) conjugated with Allophycocyanin for 30 mins on ice and washed twice and analysed by a fluorescence-activated cell sorter (FACSort, Becton Dickinson, San Jose's, USA). The data were analysed by FlowJo (Tree Star, Inc, CA, USA).

T-Cell culture medium

T-cell culture medium consisted of RPM1 1640 (CSL Ltd.) supplemented with 10% (vol/vol) heat-inactivated fetal calf serum, 2 mM L-glutamine, 2 mM sodium pyruvate, 30 μg of gentamicin/ml, 100 μg of streptomycin/ml, 100 IU of penicillin/ml, and 10^{-4} M 2-mercaptoethanol.

Cytotoxic T-cell assays

Secondary effector cells were generated either from inguinal and popliteal lymph nodes of mice that had been immunized s.c. 7 days previously with lipopeptide immunogens or from spleen cells of mice primed at least 28 days previously with the lipopeptide immunogens. Briefly, 4 X 10^7 lymph node cells or spleen cells, depleted of erythrocytes by treatment with Tris-buffered ammonium chloride (0.15 M NH_4Cl in 17 mM Tris-HCl at pH 7.2), were cultured with 10^7 irradiated (2,200 rads, ^{60}Co source) virus-infected or lipopeptide-pulsed syngeneic spleen cells in 25-cm^2 tissue culture flasks (Falcon) containing 15 ml of T-cell culture medium. The virus-infected spleen cells had been preincubated at 37°C for 30 min with 3,000 hemagglutinating units of either infectious Mem 71 or PR8 virus in 1 ml of serum-free RPM1 and washed once prior to addition to the flask. The lipopeptide-

pulsed spleen cells had been preincubated at 37°C for 60 min with 100 μg of the CTL lipopeptide/ml and also washed once prior to addition to the flask. After 5 days of culture at 37°C in a humidified atmosphere containing 5% CO_2, the cells were washed three times and used in ^{51}Cr-release assays. The ^{51}Cr-release assays were performed in triplicate as described previously (Harling-McNabb *et al, Int. Immunol. 11*, 1431-1439, 1999) by using P815 mastocytoma cells (*H-2^d*, DBA/2) as targets.

In Vivo Cytotoxic T-cell assays

The ability of various peptide-based immunogens to induce epitope-specific CTL was determined *in vivo*. Groups of three mice were inoculated intranasally with various lipopeptides in 50μl PBS and challenged with Mem71 on day 28. In order to analyze CTL determinant specific cytotoxicity *in vivo*, syngeneic spleen cells were pulsed with the CTL determinant and labelled with high intensity CFSE (2.5μM). Antigen-specific lysis was controlled by co-injecting syngeneic spleen cells labelled with low intensity CFSE (0.25μM). A mixture of 15 x 10^6 cells of each target cell population was injected intraveniously on day 4 post-infection. The mice were killed 16 hr later and spleens were analysed for the presence of CFSE-high and CFSE-low cell populations by flow cytometry. A total of 1 x 10^6 lymphocytes were analysed for each sample. Individual mice are represented by the closed squares and the bars represent the geometric mean titre.

ELISPOT assay for IFN-γ-secreting cells

CTL-specific IFN-γ-secreting cells were enumerated by an ELISPOT assay modified from that of Murali-Krishna *et al, Immunity 8*, 177-187, 1998. Flat-bottom polyvinyl chloride microtiter plates (96-well: Dynatech) were coated overnight with 50 μl of rat anti-(mouse IFN-γ) antibody (clone R4-14a2) at 5 μg/ml in PBS. Unoccupied sites on the wells were then blocked by incubation for 1 h with 10 mg of bovine serum albumin/ml in PBS, and the plates were washed three times with PBS containing 0.05% Tween 20 (PBST). Twofold dilutions of spleen or lymph node cells in T-cell medium were then added to the wells, together with 5 X 10^5 irradiated (2,200 rads, ^{60}Co source) syngeneic spleen cells from unimmunized

mice and 10 U of recombinant human interleukin-2 (Pharmingen, San Diego, Calif.)/well. Cells were incubated at 37°C in 5% CO_2 for 18 h in the presence or absence of the CTL peptide at a concentration of 1 µg of peptide/ml. Cells were then lysed and removed by rinsing the plates, initially with distilled water and then PBST. Then, 50 µl of a 1/500 dilution of biotinylated anti-(mouse IFN-γ) antibody (clone XMG 1.2; Pharmingen) was added, and the plates were incubated at room temperature for 2 h. Plates were again washed, and 50 µl of streptavidin-alkaline phosphatase (Pharmingen; 1/400 dilution in 5 mg of bovine serum albumin/ml of PBST) was added to each well; the mixtures were then incubated for a further 2 h. The plates were washed. and 100 µl of ELISPOT substrate (Sedgwick *et al, J. Immunol. Methods 57*, 301-309, 1983) containing 1 mg of BCIP (5-bromo-4-chloro-3-indolyphosphate) per ml of 2-amino-2-methyl-1-propynol buffer (Sigma) was added to each well. When blue-green spots had developed, the plates were washed with water and dried, and the spots were counted with the aid of an inverted microscope.

D1 Dendritic cell cultures

Dendritic cells (DC) were cultured in medium based on complete IDDM. This consisted of Iscove's Modified Dulbecco's Medium (IMDM) containing 25 mM HEPES and without alpha-thioglycerol or L-glutamine (JRH Bioscience, Lenexa, USA), supplemented with 10% (v/v) heat inactivated (56°C, 30 min) foetal calf serum (CSL Ltd., Parkville, Victoria, Australia), gentamicin (24 µg/mL), glutamine (2 mM), sodium pyruvate (2 mM), penicillin (100 IU/mL), streptomycin (180 µg/mL) and 2-mercaptoethanol (0.1 mM). For DC generation complete IMDM was further supplemented with 30% supernatant from cultured NIH/3T3 cells and 5% GM-CSF in the form of a supernatant from Ag8653 cells transfected with the GM-CSF gene (DC medium).

The culture method for immature dendritic cells was adapted from Winzler *et al., J. Exp Med. 185*, 317 (1997). Spleen cells from a BALB/c mouse were seeded at 1.5×10^6 cells per 55 mm dish (Techno-Plas, S.A., Australia) in 3 ml DC medium and incubated at 37°C with 5% CO_2. All the equipment used for culturing was pyrogen free. The medium was changed every 4 days and all cells returned to the

dish. On day 12, both suspended and weakly adherent cells were collected by forcefully pipetting and then aspirating the medium. The procedure was repeated with 2 ml of PBS. The remaining strongly adherent cells were discarded. The collected cells were pelleted by centrifugation and reseeded into a new dish. Cells were subsequently maintained on a 4 day alternating cycle of media change and passage. After 1 month of continuous culturing, the floating and semi-adherent cells took on the appearance and staining characteristics of immature DC and are referred to as D1 cells. Under these passage conditions the majority of cultured D1 cells maintain an immature phenotype characterized by an intermediate expression level of cell surface MHC class II molecules.

Flow cytometric analysis of D1 cells

D1 cells (1 x 10^5 cells per sample) were seeded in a new Petri dish with 1 mL of DC media and incubated with 0.0045 nmole of lipopeptide, dissolved in complete IMDM medium. Lipopolysaccharide (LPS) purified from E. coli serotype O111:B4 (Difco, Detroit, Michigan, USA, was used at 5 μg/mL as a positive control for DC maturation. After overnight incubation, the cells were harvested and washed once with PBS with 1% FCS. To prevent non-specific binding to FCγRII/III, the cells were pre-incubated with 20 μL of normal mouse serum for 5 mins at room temperature. The cells were then exposed to FITC-conjugated monoclonal antibody 14-4-4S (IgG_{2a} , anti-I-$E^{k,d}$; Ozato *et al., J. Immunol.,124,* 533 (1980)) for 30 min on ice. Monoclonal antibody 36/1 (Brown *et al., Arch Virol 114:* 1, 1990), which is specific for the antigen of influenza virus from which the T-helper epitope is derived, was used as an isotype control. All antibodies were used at 2.5 μg/mL. The samples were washed once with PBS containing 1% FCS and fixed with PBS containing 4% paraformaldehyde on ice for 15 minutes. Flow cytometry analysis was performed using a FACSort (Becton Dickinson, San Jose, USA) and the data were analysed using FlowJo software (Tree Star, Inc., San Carlos,CA, USA).

Human Dendritic cell cultures

Generation of monocyte-derived dendritic cells

Peripheral blood mononuclear cells (PMBCs) were prepared from buffy coat preparations obtained from blood donors (Red Cross Blood Bank, Melbourne, Australia) by Ficoll Paque (Amersham Pharmacia, Sweden) gradient separation. The cells were washed three times in PBS and incubated with optimal amounts of murine anti-CD14 hybridoma supernatant (3C10, American Type Culture Collection) for 45 minutes on ice. After two washes, cells were further incubated with goat anti-murine IgG microbeads (Miltenyi Biotech, Germany) according to the manufacturer's protocol. $CD14^+$ monocytes were then positively selected by affinity purification using a magnet-activated cell sorting (MACS) column. Immature DC were generated by culturing the monocytes in GM-CSF and IL-4 (40ng/ml and 20ng/ml, respectively [Schering Plough, USA]) supplemented RPMI-1640 (Gibco, USA) containing 10% FCS (CSL, Australia), 2mmol/L glutamine, 2mmol/L sodium pyruvate, 100 U/ml penicillin, 100µg/ml streptomycin, 30µg/ml gentamicin and 0.1mmol/L 2-mercaptoethanol. Cells were cultured for 5 days before use with half volume changes of media every 2 days.

Measurement of DC maturation

The ability of peptide and lipopeptide-based immunogens to up-regulate the expression of MHC class II, CD83 and CD86 on human monocyte-derived dendritic cells was determined by incubating 5 x 10^5 cells per ml for 2 days in medium supplemented with GM-CSF and IL-4 and either LPS (5µg/mL), non-lipidated peptide [Th]-*Lys*-[CTL] (5µg/mL) or lipopeptide [Th]-*Lys*(Pam_2Cys-Ser-Ser)-[CTL] (5µg/mL) for 48 hours. Phenotypic analysis of surface markers was performed by staining with fluorochrome-conjugated monoclonal antibodies to HLA-DR (G46-6 [L243]), CD83 (HB15e), CD86 (Cat. No. 2331 [FUN-1]) and appropriate isotype matched antibodies (MOPC-21 and G155-178) from Becton Dickinson (USA), according to the manufacturer's protocols. Cells were then washed, fixed in 1% formaldehyde and analysed on a flow cytometer. The histograms are representative of large granular cells gated on the forward and side scatter dot plot. The shaded regions of the histograms and the associated

numerical values identify the percentage of cell populations expressing high levels of CD83, CD86 or HLA-DR.

EXAMPLE 2

Immunogenicity of lipopeptides comprising CTL epitopes from influenza virus

Lipopeptides having a CTL epitope from influenza virus and in particular the lipopeptides [Th]-*Lys*(Pam_2Cys-Ser-Ser)-[CTL] and [Th]-*Lys*(Pam_3Cys-Ser-Ser)-[CTL], which comprise the amino acid sequence set forth in SEQ ID NO: 4 were tested for their ability to induce enhanced CTL-mediated viral clearance and to enhance dendritic cell maturation. As a negative control, a non-lipidated peptide having the amino acid sequence of SEQ ID NO: 4 was used in all experiments.

Viral Clearance

The lipopeptides elicited a higher level of viral clearance than non-lipidated peptides (Figures 3, 4a). Viral load in the lungs of mice primed with the lipopeptides and challenged with infectious Mem 71 virus 9 days later was reduced by 95% ([Th]-*Lys*(Pam_3Cys-Ser-Ser)-[CTL]; Figure 3) or 99% ([Th]-*Lys*(Pam_2Cys-Ser-Ser)-[CTL]; Figure 3) compared to samples from mice immunized with PBS alone. In contrast, non-lipidated peptide achieved only a 65% reduction in viral load ([Th]-*Lys*-[CTL]; Figure 3). Enhanced viral clearance was also observed in lipopeptide-inoculated animals that had been challenged with Mem 71 virus 28 days after the initial inoculation. In contrast, the ability to clear virus is significantly weaker at this time point in mice inoculated with the non-lipidated peptide.

As shown in Figure 4b, there was also enhanced $CD8^+$ T cell activation in immunized mice receiving the lipopeptides referred to in the legend to Figure 2, compared to mice receiving only non-lipidated peptide or PBS as seen by the number of CD8+ T clls found in the BAL fluids.

Dendritic Cell maturation

The priming of naïve CD4+ T cells and CD8+ T cells in secondary lymphoid organs by dendritic cells is preceded by maturation of DC upon exposure to antigen epitope. This maturation is characterised by up-regulation of MHC products and co-stimulatory molecules on the DC surface. We therefore determined whether the various peptides and lipopeptides could differentially activate dendritic cells in an attempt to explain the different immunogenic properties of these vaccine candidates.

The results of experiments in which a line of immature DC, D1 cells, were exposed to peptides, stained for surface expression of MHC class II molecules then analysed by flow cytometry, demonstrated that there was enhanced maturation of dendritic cells following their exposure to the peptides [Th]-*Lys*(Pam_3Cys-Ser-Ser)-[CTL] or [Th]-*Lys*(Pam_2Cys-Ser-Ser)-[CTL] compared to [Th]-*Lys*-[CTL] peptide or medium alone (Figure 4c).

[Th]-*Lys*(Pam_2Cys-Ser-Ser)-[CTL] was the most effective and the non-lipidated peptide [Th]-*Lys*-[CTL] was the least effective in causing maturation of DC, with [Th]-*Lys*(Pam_3Cys-Ser-Ser)-[CTL] being nearly as effective as [Th]-*Lys*(Pam_2Cys-Ser-Ser)-[CTL] (Figure 4c). The ability of the lipidated peptide [Th]-*Lys*(Pam_2Cys-Ser-Ser)-[CTL] to up-regulate class II expression was the same as for bacterial lipopolysaccharide (LPS). The non-lipidated peptide was unable to induce maturation of D1 cells greater than about 26%, a level that occurs spontaneously in culture. The relative abilities of these lipopeptides to induce maturation of D1 cells directly reflected their ability to induce CTL-mediated viral clearing responses and CD8+ T cells in the BAL.

Effects of different lipids on cytotoxicity and T cell proliferation in vitro and in vivo

The effects of conjugating different lipids, including Pam_1Cys, Pam_2Cys, Pam_3Cys, palmitic acid and cholesterol, to the peptide immunogen were also determined.

As shown in Figure 5, viral load in the lungs of mice primed with Pam_2Cys-containing lipopeptides were lower than for mice primed with lipopeptides comprising the other lipids tested, suggesting that Pam_2Cys is preferred for conferring protection against virus. All lipids however, offered some protection against virus. This effect was also reflected in the IFN-gamma CD8+ T cell count (Figure 6). Collectively, these data suggest that it is important to attach the lipid to the cysteine glycerol residue, as in the [Th]-*Lys*(Pam_2Cys-Ser-Ser)-[CTL] epitope structure, for maximum cytotoxic effect.

In tetramer assays, the highest number of tetramer positive CD8+ T cells per lung were observed for lipopeptides wherein the lipid moiety was added to the epsilon amino group of an internal lysine residue (e.g., lipopeptides [Th]-*Lys*(Pam_1Cys-Ser-Ser)-[CTL], [Th]-*Lys*(Pam_2Cys-Ser-Ser)-[CTL], [Th]-*Lys*(Pam_3Cys-Ser-Ser)-[CTL], and [Th]-*Lys*($Chol_2Lys$-Ser-Ser)-[CTL] in Figure 7) compared to non-lipidated peptide or lipopeptide having lipid added to the N-teminus of the peptide (e.g., construct $Pal_2LysLys$[Th]-[CTL] in Figure 7). These data also confirm that the positioning of the lipid internal to the peptide, by attachment to the epsilon amino group of an internal lysine residue, enhances cytotoxic activity of the CTL epitope.

To analyze CTL determinant specific cytotoxicity *in vivo*, mice were inoculated intranasally with 9nmoles of various lipopeptides in PBS and challenged with Mem71 virus on day 28. CTL determinant-specific cytotoxicity *in vivo* was measured using syngeneic spleen cells pulsed with the CTL determinant and labelled with high intensity CFSE. Non-pulsed spleen cells labelled with low intensity CFSE were used as a control. A mixture of cells of each target cell population was injected intraveniously on day 4 post-infection. The mice were killed 16 hr later and spleens were analysed for the presence of CFSE-high and CFSE-low cell populations by flow cytometry. A total of 1×10^6 lymphocytes were analysed for each sample. Data in Figure 8 is a graphical representation showing

cytotoxic T cell activity in naïve mice. Figure 9 indicates that the lipopeptide [Th]-*Lys*(Pam_2Cys-Ser-Ser)-[CTL] comprising the $CD4^+$ T-helper epitope set forth in SEQ ID NO: 1 and the *H-2^d*-restricted CTL epitope set forth in SEQ ID NO: 2, induced significant cytotoxicity *in vivo*.

As shown in Figure 10, lipopeptides have higher activity than non-lipidated peptide, with the lipopeptides designated [Th]-*Lys*(Pam_1Cys-Ser-Ser)-[CTL], [Th]-*Lys*(Pam_2Cys-Ser-Ser)-[CTL] and [Th]-*Lys*(Pam_3Cys-Ser-Ser)-[CTL] providing a marked enhancement of specific lysis *in vivo* compared to the non-lipidated peptide [Th]-*Lys*-[CTL] and other lipopeptides tested. These data again confirm that positioning of the lipid internal to the peptide, by attachment to the epsilon amino group of an internal lysine residue, enhances cytotoxic activity of the CTL epitope *in vivo*.

EXAMPLE 3

Immunogenicity of lipopeptides comprising a CTL epitope from *L. monocytogenes*

A lipopeptide having a CTL epitope from *L. monocytogenes* and in particular the lipopeptide [P25]-*Lys*(Pam_2Cys-Ser-Ser)-[LLO91-99] comprising the amino acid sequence set forth in SEQ ID NO: 175 was tested for its ability to induce a CD8+ T cell response, and to protect against a challenge with *L. monocytogenes*. As a negative control, PBS or a non-lipidated peptide having the amino acid sequence of SEQ ID NO: 175 was used in all experiments. Isolated bacteria were used as a positive control.

IFN-γ production by splenocytes

The lipopeptide tested in this study induced a specific CD8+ T cell response against the immunizing CTL epitope, as evidenced by the enhanced number of IFN-γ producing splenocytes present in mice immunized with lipidated peptide relative to non-lipidated peptide. Mice immunized with 9nmoles lipidated peptide vaccine [P25]-*Lys*(Pam_2Cys-Ser-Ser)-[LLO91-99] comprising the amino acid sequence set forth in SEQ ID NO: 175 produced about 15-fold more IFN-γ

producing cells per million splenocytes than mice receiving non-lipidated peptide or a PBS control, indicating an enhanced activation of IFN-γ producing CD8+ T cells in the mice receiving the lipidated peptide (Figure 11).

Protection against challenge with isolated bacteria

Data in Figure 12 indicate that the lipidated [P25]-*Lys*(Pam_2Cys-Ser-Ser)-[LLO91-99] peptide successfully provides protection against a subsequent challenge with whole bacteria. A significantly enhanced protection was also observed in mice immunized with the lipidated [P25]-*Lys*(Pam_2Cys-Ser-Ser)-[LLO91-99] peptide relative to mice immunized with non-lipidated [P25]-*Lys*-[LLO91-99] peptide or PBS (i.e. non-immunized mice).

EXAMPLE 4
Protection against challenge with tumour cells

Protection against challenge with Melanoma cells

The ability of the lipopeptide vaccine containing the ovalbumin CTL epitope (SIINFEKL) to induce protection against melanoma cells expressing this CTL epitope (B16-OVA cells) was assessed. IFN-γ production was determined in mice inoculated with lipopeptide comprising a CDV-F T-helper epitope (P25) and a CTL epitope (SIINFEKL) of ovalbumin linked via the epsilon amino group of an internal lysine residue positioned between said epitopes to Pam_2Cys (i.e. the peptide [P25]-*Lys*(Pam_2Cys-Ser-Ser)-[SIINFEKL] listed in Figure 2 and based upon SEQ ID NO: 174). C57BL/6 mice were vaccinated with 20 nmoles lipidated peptide [P25]-*Lys*(Pam_2Cys-Ser-Ser)-[SIINFEKL], non-lipidated peptide [P25]-*Lys*-[SIINFEKL] or with PBS subcutaneously in the base of the tail. Mice were then challenged subcutaneously on the back 14 days later with B16-OVA cells. Splenocytes were obtained from the inoculated animals and stimulated *in vitro* with the CTL epitope having the sequence SIINFEKL and the number of IFN-γ producing cells per 1,000,000 splenocytes was measured. Data show enhanced numbers of IFN-γ producing cells for mice inoculated with lipopeptide (Table 1),

indicating an enhanced ability of the lipopeptides to activate T cells relative to non-lipidated peptide.

Importantly, control of tumour growth was elicited by immunisation with lipopeptide compared to mice immunized with the non-lipidated peptide [P25]-*Lys*-[SIINFEKL] or PBS alone (Figure 13). No tumour growth was observed over a 15 day period in mice immunised with [P25]-*Lys*(Pam_2Cys-Ser-Ser)-[SIINFEKL]. Conversely, tumours of greater than 75mm^2 in diameter were observed in mice immunised with [P25]-*Lys*-[SIINFEKL] or PBS alone. Together, these data confirm the protective ability of the lipopeptide compared to non-lipidated peptide in protection against tumours.

TABLE 1

Numbers of IFN-γ secreting splenocytes in representative melanoma samples receiving [P25]-*Lys*(Pam_2Cys-Ser-Ser)-[SIINFEKL] lipopeptide compared to non-lipidated [P25]-*Lys*-[SIINFEKL] peptide or PBS

	PEPTIDE/LIPOPEPTIDE IMMUNOGEN		
No. IFN-γ secreting splenocytes per 10^6 splenocytes	**[P25]-*Lys*(Pam_2Cys-Ser-Ser)-[SIINFEKL**	**[P25]-*Lys*-[SIINFEKL]**	**PBS**
	284	18	5
	205	14	0
	192	10	0
Average	227	14	3
Std. deviation	49	4	24

Protection against challenge with Lewis Lung tumour cells

The ability of the lipopeptide to provide protection against Lewis Lung tumor development in animals *in vivo* was also tested. Mice were injected with 3x10^4 Lewis Lung tumour cells transfected with ovalbumin and therefore expressing the

CTL epitope SIINFEKL (Nelson *et al., J Immunol. 166*: 5557-5566, 2001). Four days after receiving tumour cells, animals were inoculated subcutaneously in the base of the tail with 20nmoles lipidated peptide [P25]-*Lys*(Pam_2Cys-Ser-Ser)-[SIINFEKL], or alternatively, non-lipidated peptide [P25]-*Lys*-[SIINFEKL] or PBS. A second and similar dose of immunogen was administered eleven days after receiving the tumour cells. Data in Figure 14 indicate that the percentage of animals with fewer lesions developing was significantly higher for animals receiving the lipopeptide compared to animals receiving the non-lipidated peptide or PBS. As shown in Figure 15, animals receiving the lipopeptide immunogen also survived for longer than those receiving the non-lipidated peptide or PBS. These data further confirm the protective ability of the lipopeptide compared to non-lipidated peptide for protection against tumours.

EXAMPLE 5

Enhanced expression of MHC class II, CD83 and CD86 on human dendritic cells following administration of a lipopeptide comprising a CDV-F T-helper epitope and a CTL epitope from hepatitis C virus

The lipopeptide [P25]-*Lys*(Pam_2Cys-Ser-Ser)-[HCV] described in the legend to Figure 2 was tested for its ability to up-regulate the expression of MHC class II, CD83 and CD86 on human dendritic cells. Human monocyte-derived dendritic cells were incubated with media alone, LPS (5μg/mL), non-lipidated peptide [P25]-*Lys*-[HCV] (5μg/mL) or lipopeptide [P25]-*Lys*(Pam_2Cys-Ser-Ser)-[HCV] (5μg/mL) for 48 hours before staining with FITC-conjugated antibodies for HLA-DR, CD83 and CD86 before analysis by flow cytometry. Data shown in Figure 16 demonstrate a higher percentage of dendritic cell populations that express HLA-DR, CD83 and CD86 antigens on their cell surface are present following treatment with lipidated peptide than following treatment with non-lipidated peptide or PBS alone. The ability of the lipopeptide to induce maturation of human dendritic cells directly reflected the immunogenic ability of the lipopeptide compared to the non-lipidated peptide, providing a possible mechanism for immunogenicity.

EXAMPLE 6
Discussion

In this study we describe the assembly of a variety of lipopeptide immunogens composed of a $CD4^+$ T cell epitope, a $CD8^+$ CTL epitope and Pam_3Cys or Pam_2Cys linked thereto via the epsilon amino group of an internal lysine residue.

The precise nature of the lipid moiety in generating an immune response was not shown to be critical, because a range of lipids, including cholesterol, palmitic acid, Pam_1Cys, Pam_2Cys, and Pam_3Cys were shown to successfully elicit T cell proliferation and cytotoxicity. However, significant differences were observed in terms of protection and IFN-gamma production, at least in the case of lipopeptides directed against influenza virus, suggesting that lipid structure may be an important consideration *in vivo*. In particular, at least for vaccines incorporating the influenza virus CTL epitope, Pam_2Cys linked to the epsilon amino group of an internal lysione residue in the peptide weremost effective in conferring protection, suggesting that a linkage to the cysteine glycerol is preferred.

The lipopeptides of the invention are effective in enhancing the CD8+ T cell responses of immunized animals against bacterial and viral pathogens and also against tumour cells. Given the success of the self-adjuvanting peptides exemplified herein to protect against viral and bacterial pathogens as well as tumour cells, it is reasonable to expect that this technology is generally applicable to a wide range of vaccination protocols.

Insertion of serine residues between the lipid moiety and the peptide sequence does not adversely affect the potency of the resulting Pam_2Cys-containing immunogens.

The lipopeptides can trigger an immune response in the absence of additional adjuvant and can be delivered by both parenteral and non-parenteral routes, particularly intranasally.

Taken together, the data provided herein demonstrate that placement of a wide range of lipids, including but not limited to Pam_2Cys and Pam_3Cys, between the CTL epitope and the T helper epitope, at the approximate centre of a totally synthetic peptide vaccine increases the immunogenicity of the vaccine.